Herpes Simplex Virus 1 ICP34.5 Alters Mitochondrial Dynamics in Neurons

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ABSTRACT Expression of viral genes and activation of innate antiviral responses during infection result in an increase in reactive oxygen species (ROS) and toxic by-products of energy metabolism which can lead to cell death. The mitochondrion and its associated proteins are crucial regulators of these responses and related pathways such as autophagy and apoptosis. Through a mass spectrometry approach, we have shown that the herpes simplex virus 1 (HSV-1) neurovirulence- and autophagy-modulating protein ICP34.5 interacts with numerous mitochondrion-associated factors. Specifically, we showed that amino acids 68 to 87 of ICP34.5, the domain that binds beclin1 and controls neurovirulence, are necessary for interactions with PGAM5, KEAP1, and other regulators of the antioxidant response, mitochondrial trafficking, and programmed cell death. We further show that while this domain interacts with multiple cellular stress response factors, it does not alter apoptosis or antioxidant gene expression. That said, the attenuated replication of a recombinant virus lacking residues 68 to 87 (termed $\Delta 68-87$) in primary human fibroblasts was restored by addition of ferric nitrate. Furthermore, in primary mouse neurons, the perinuclear localization of mitochondria that follows infection with HSV-1 was notably absent following $\Delta 68-87$ infection. Through this 20-amino-acid domain, ICP34.5 significantly reduces mitochondrial motility in axons of neurons. We propose the hypothesis that ICP34.5 promotes perinuclear mitochondrial localization by modulating transport of mitochondria through interaction with PGAM5. These data expand upon previous observations of altered mitochondrial dynamics following alphaherpesvirus infections and identify a key determinant of this activity during HSV-1 infections.

IMPORTANCE Herpes simplex virus persists lifelong in neurons and can reactivate to cause recurrent lesions in mucosal tissues. A key determinant of virulence is the viral protein ICP34.5, of which residues 68 to 87 significantly contribute to neurovirulence through an unknown mechanism. Our report provides evidence that residues 68 to 87 of ICP34.5 are required for binding mitochondrion-associated factors. These interactions alter mitochondrial dynamics in neurons, thereby facilitating viral replication and pathogenesis.

KEYWORDS herpes simplex virus, mitochondria, neurovirulence

The neurotropic herpes simplex viruses 1 (HSV-1) and 2 (HSV-2) are widely prevalent and persist lifelong (1–4). HSV-1 lytic replication in mucosal membranes is followed by infection of innervating sensory nerves (5). The virus travels through axons to the neuronal cell bodies (soma) housed in sensory and sympathetic ganglia, where it establishes a dormant state, termed latency (6–10). Periodically, the virus reactivates to produce progeny virions which traffic to the site of primary infection, where they may cause viral lesions and disseminate (11). Viral infection is detected by the cell through pattern recognition receptors (PRRs) which detect conserved structures on the virus

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called pathogen-associated molecular patterns (PAMPs) (12). Concerted efforts of the innate immune response induce a myriad of defenses, including upregulation of antiviral genes, such as interferons (IFNs), to prime the immune system and prevent viral spread (13, 14). Of note, type I IFNs (IFN- α and IFN- β) act in an autocrine and paracrine fashion to amplify and propagate the antiviral state in infected and neighboring naive cells and are crucial in protecting neurons during HSV infection (15, 16). IFN- α and - β promote a positive-feedback loop by binding to IFN- α/β receptors on the plasma membrane which activate a cascade of antiviral signals leading to intensification of IFN and interferon-stimulated gene (ISG) production (17–19). In addition, viral double-stranded RNA (dsRNA) triggers dsRNA-dependent protein kinase (PKR) to phosphorylate and inactivate eukaryotic initiation factor 2 alpha (eIF2 α), leading to translational shutoff and furthering the antiviral state of the cell (20, 21).

To combat the host response to infection, HSV-1 has evolved numerous factors to manipulate or evade the immune system. In particular, the multifunctional infected-cell protein 34.5 (ICP34.5) blocks many facets of the antiviral response. HSV-1 ICP34.5 is a 248-amino-acid protein encoded by the leaky-late gene, γ 34.5 (22). Its C terminus is homologous to the human growth arrest and DNA damage 34 (GADD34) protein, which recruits protein phosphatase 1-alpha (PP1 α) to dephosphorylate eIF2 α , thereby allowing viral protein synthesis to persist (23–25). In addition, ICP34.5 facilitates viral nuclear egress by recruiting complement component 1 Q subcomponent-binding protein (C1QBP/p32) to the inner nuclear membrane, leading to rearrangement of the nuclear lamina (26). It also plays a role in blocking the IFN pathway and binds beclin1 to inhibit autophagy, a process involved in recycling or degrading cellular and microbial components (27–30). Amino acids (aa) 68 to 87 of ICP34.5 are required to bind beclin1, and viruses lacking this domain are neuroattenuated (27). However, the mechanism by which this domain of ICP34.5 modulates neurovirulence has yet to be elucidated.

Establishment of the antiviral state and production of ISGs comes at a high metabolic cost. During infection, the bioenergetics required to orchestrate an immune response are also to the detriment of virion production (31). The summation of energy produced in the cell is achieved by glycolysis in the cytoplasm and the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS) in the mitochondria (32). Heightened OXPHOS activity produces reactive oxygen species (ROS), which can accumulate and lead to programmed cell death. In response to oxidative damage, the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) binds antioxidant response element (ARE)-containing promoters to upregulate antioxidant genes (33). During homeostasis, Nrf2 is bound to its negative regulator, KEAP1 (Kelch-like ECH-associated protein 1), and tethered to the mitochondria by PGAM5 (phosphoglycerate mutase family member 5) (34). In addition, the mitochondria and their associated proteins regulate multiple cellular stress responses. The mitochondrial proteins Bcl-2 and Bcl-xL regulate apoptosis by controlling mitochondrial membrane integrity and autophagy by interacting with beclin1 (35–37). Consequently, appropriate distribution and regulation of mitochondria are crucial for cellular function, and viruses have evolved means to commandeer these processes (reviewed in reference 38). In neurons, mitochondria are especially important due to the disproportionately high metabolic costs associated with neuronal processes (39). For example, mitochondria are recruited to sites of synapse formation and activity as well as axonal termini to provide ATP and calcium buffering during neurotransmission (40–42). Consequently, alphaherpesviruses have evolved mechanisms to alter these biological functions to promote infection. The neurotropic porcine herpesvirus pseudorabies virus (PRV) disrupts mitochondrial motility and morphology in neurons by altering intracellular calcium through glycoprotein B (gB) (43). HSV-1 and -2 cause altered mitochondrial movement and perinuclear localization in neurons and keratinocytes, by an unknown mechanism (43–45). Through the exonuclease activity of U_L12.5, HSV-1 infection causes the rapid depletion of mitochondrial DNA (46, 47). This activity is likely to prevent DNA sensing and amplified antiviral signaling through the cGAS/STING pathway. Infection with the betaherpesvirus human cytomegalovirus (HCMV) increases oxidative phosphorylation and altered

FIG 1 Map of ICP34.5 function domains and recombinant viruses. A schematic diagram of ICP34.5 with relevant binding domains is shown. The region from aa 68 to 87 binds to multiple proteins, but for clarity only beclin1 is indicated. Below are shown viral variants, indicating the limits of the in-frame deletions engineered into the ICP34.5 open reading frame of HSV-1 strain 17.

mitochondrial morphology and function (48). Identifying the means by which human alphaherpesviruses reorganize mitochondria will provide important insights into the interplay of viral infection with the disruption and maintenance of cellular homeostasis.

Our studies were designed to elucidate the mechanism by which HSV-1 mutants lacking aa 68 to 87 of ICP34.5 ($\Delta 68-87$) are attenuated. Here, we show by coimmunoprecipitation and tandem mass spectrometry (MS/MS) that aa 68 to 87 of ICP34.5 are required to bind PGAM5, KEAP1, and other factors involved in mitochondrial dynamics. PGAM5 plays a key role in programmed cell death, the antioxidant response, and mitochondrial trafficking (49–53). Importantly, PGAM5 regulates retrograde trafficking of mitochondria through a complex with KEAP1 and Nrf2 (50). Although we did not observe differences in global antioxidant response, apoptosis, or cell death in human foreskin fibroblasts (HFFs), aa 68 to 87 were required to alter mitochondrial motility and localization in HSV-infected primary mouse neurons. In addition, the attenuated replication of $\Delta 68-87$ *in vitro* was partially restored in the presence of ferric nitrate, an iron salt involved in reduction-oxidation (redox) buffering. Taken together, our data are consistent with the hypothesis that ICP34.5 recruits mitochondria to the soma of neurons to make it bioenergetically favorable for efficient virion production.

RESULTS

Identification of HSV-1 ICP34.5 binding partners by mass spectrometry. Using expression constructs and recombinant viruses with mutations in ICP34.5, we wished to elucidate host pathways that impact the phenotype of $\Delta 68-87$, a virus which lacks aa 68 to 87 of ICP34.5 (Fig. 1) (27, 28, 54). To date, ICP34.5 has been shown to interact with beclin1, TBK1, C1QBP/p32, PCNA, eIF2 α , and PP1 α (24, 26, 27, 29, 55, 56). In an unbiased approach to further our knowledge of the ICP34.5 interactome, we utilized tandem mass spectrometry to elucidate protein complexes associated with ICP34.5. Previously, we generated hemagglutinin (HA)-tagged expression constructs with various in-frame mutations in ICP34.5 (28). These were expressed in HEK-293T cells and immunoprecipitated to confirm equal expression and precipitation (Fig. 2A). In parallel, immunoprecipitated samples were analyzed by mass spectrometry to identify protein interactions which were absent in p $\Delta 68-87$ -HA but present in full-length ICP34.5 (p34.5-HA) (Fig. 2B; see Data Set S1 in the supplemental material). Strikingly, we observed a high number of mitochondrion-associated proteins bound to ICP34.5. More than 50 proteins were reproducibly lower in abundance in p $\Delta 68-87$ -HA than in p34.5-HA (displayed in blue in Fig. 2B). We compared a selection of these proteins to known ICP34.5 interactions (Fig. 2C). We used an empty vector (pCAGGS) as an internal control for background signal. The abundances of ICP34.5 and known partners, C1QBP/p32 and PP1 α , were similar for p $\Delta 68-87$ -HA and p34.5-HA, although unexpectedly the abundances of

FIG 2 Identification of ICP34.5 binding partners by mass spectrometry. (A) Representative Coomassie blue-stained polyacrylamide gel showing amounts of protein corresponding to ICP34.5 and ICP34.5 variants captured by coimmunoprecipitation. (B and C) Volcano plot of intensity-based absolute quantification (iBAQ) values generated by tandem mass spectrometry analysis of coimmunoprecipitated proteins which bind full-length ICP34.5 p34.5-HA but not pΔ68-87-HA (blue dots) (B) and comparison of selected known and novel ICP34.5 interactions (C). HEK-293T cells expressing p34.5-HA or the ICP34.5 variant pΔ68-87-HA were coimmunoprecipitated using an anti-HA.11 antibody. Eluates were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and searched using COMET against human and HSV-1 proteome sequence databases (UniProt). The data points represent means $\pm$ standard errors of the means (SEM) and are a summation of the results of three independent experiments. Statistical significance was determined by the two-tailed unpaired *t* test. *, $P < 0.05$; **, $P > 0.01$; ns, not significant. (D) Western blots of coimmunoprecipitations showing interaction of ICP34.5 variants with endogenous PGAM5. HEK-293T cells were transfected with HA-tagged ICP34.5 variants or empty vector control. At 24 h posttransfection, lysates were immunoprecipitated using an anti-HA.11 antibody. The precipitates and lysates were analyzed by immunoblotting using anti-PGAM5, anti-ICP34.5, or anti-HA.11 antibodies. The LC-MS/MS immunoblots are representative of the results of three independent coimmunoprecipitation experiments.

eIF2 α were slightly lower for pΔ68-87-HA immunoprecipitations. Abundances of the multifunctional proteins PGAM5 and KEAP1, the antiapoptosis factor CAAP1, and ECHB, an enzyme involved in lipid metabolism, were all significantly decreased for pΔ68-87-HA compared to p34.5-HA (Fig. 2C). We focused on PGAM5 due to its overlapping interaction with many of the proteins identified by mass spectrometry. PGAM5 is a serine/threonine/histidine phosphatase which regulates multiple aspects of mitochondrial dynamics, including the antioxidant response, programmed cell death, and mitochondrial trafficking (49–52). In order to confirm ICP34.5-PGAM5 binding, we performed Western blotting on immunoprecipitated lysates from cells expressing ICP34.5 and its variants (Fig. 2D). Full-length ICP34.5 bound both isoforms of PGAM5, as shown by the double-banding pattern, and these interactions were lost in pΔ68-87-HA- and pΔ68-107-HA-expressing samples. A 20-aa deletion immediately downstream of residue 87, pΔ87-106-HA, did not disrupt interaction with PGAM5. Taken together, these results

expand upon known partners of ICP34.5 and provide an interactome to further dissect potential pathways utilized by HSV-1 that may be important during infection.

Replication of $\Delta 68-87$ is enhanced by ferric nitrate. In mice, HSV-1 strains lacking aa 68 to 87 of ICP34.5 exhibit diminished neurovirulence and viral replication *in vivo* relative to wild-type viruses (27, 28, 57). In contrast, previous studies observed no difference in replication in primary fibroblasts (58) and a 10-fold replication defect in $\Delta 68-87$ compared to wild-type HSV-1 infection in cultured neurons primed with IFN- β (59–61). The abundance of antioxidant and bioenergetic factors identified by mass spectrometry (Fig. 2B) provoked the hypothesis that the attenuated phenotype of $\Delta 68-87$ *in vivo* is complemented by some component of cell culture. Here, we examine replication of $\Delta 68-87$ using primary human foreskin fibroblasts (HFFs) treated with IFN- β or vehicle control prior to infection with recombinant viruses. In cells infected in Dulbecco's modified Eagle's medium (DMEM), IFN- β pretreatment resulted in failure of $\Delta 34.5$ to replicate and a slight reduction in replication of $\Delta 68-87$ compared to wild-type strain 17 virus (Fig. 3A). These results recapitulate similar experiments in mouse neurons and human and mouse fibroblasts (28, 58–60). However, when cells were infected in minimum essential medium (MEM), IFN- β pretreatment led to a 100-fold reduction in replication of $\Delta 68-87$ compared to wild-type virus (Fig. 3B). Replication of $\Delta 34.5$ was similar in both DMEM and MEM. We therefore wished to determine which growth medium component(s) was responsible for the observed differences in $\Delta 68-87$ replication. First, we tested HFF cell viability in DMEM and MEM and observed no differences (data not shown). DMEM and MEM differ in the concentration and composition of inorganic salts, amino acids, and vitamins. In a reductionist approach, we used MEM as the base medium and added components which were absent in MEM but present in DMEM. Our experiments showed that the iron salt ferric nitrate, when added to MEM, restored replication of $\Delta 68-87$ to near-wild-type levels in IFN- β -primed HFF cells (Fig. 3C). Similar to viral growth in DMEM versus MEM, addition of ferric nitrate to MEM in the absence of IFN- β pretreatment had no impact on replication (Fig. 3D). Given that aa 68 to 87 are involved in the inhibition of autophagy (27) and that ferric nitrate reduced some of the interferon sensitivity of $\Delta 68-87$ (Fig. 3C), we tested the effects of ferric nitrate on autophagy and IFN- β signaling. As expected, IFN- β treatment increased levels of phosphorylated STAT1 and total STAT1 compared to those in vehicle control-treated HFFs (Fig. 3E). Addition of ferric nitrate, however, did not alter the ability of the cells to respond to IFN- β treatment as measured by phosphorylation and abundance of STAT1. In addition, ferric nitrate addition did not impact the autophagy marker p62 or LC3-I-to-LC3-II conversion (Fig. 3E). Taken together, these results indicate that the replication of $\Delta 68-87$ in the presence of IFN is restored by addition of ferric nitrate, a metabolic cofactor and molecule involved in protecting cells from oxidative damage.

Residues 68 to 87 of ICP34.5 do not impact global antioxidant response or apoptosis. Previous work has identified PGAM5 as a key factor in cellular regulation of oxidative stress and numerous forms of programmed cell death (49, 51, 52, 56, 62). PGAM5 is tethered to the outer mitochondrial membrane (OMM) and interacts with antioxidant response regulators KEAP1 and Nrf2 and antiapoptosis factor Bcl-xL (34, 51, 63). Additionally, the same region of ICP34.5 that interacts with PGAM5 and KEAP1 interacts with beclin1, an autophagy-related protein which has antiapoptotic attributes and binds Bcl-xL at the OMM (27, 64–67). It has therefore been suggested that ICP34.5 modulates apoptosis through interaction with beclin1 and others through this domain (61). We wished to determine the role of aa 68 to 87 in the context of antioxidant response and apoptosis during infection. Primary HFF cells grown with or without ferric nitrate and pretreated with IFN- β or vehicle control were infected with HSV-1 variants and analyzed by Western blotting (Fig. 4A). Interestingly, we did not observe a difference in the activated form of the antioxidant transcription factor phosphorylated Nrf2 (P-Nrf2) or its downstream antioxidant response gene product superoxide dismutase 2 (SOD2-Mn) during $\Delta 68-87$ compared to wild-type strain 17 infection (Fig. 4A,

FIG 3 Ferric nitrate rescues replication of $\Delta 68-87$ mutant virus. (A and B) Multiple-step growth curves showing replication of $\Delta 34.5$, $\Delta 68-87$, or wild-type HSV-1 strain 17 in primary HFFs in DMEM (A) or MEM (B) growth medium. The HFFs were treated with vehicle control or 100 IU/ml IFN- β for 18 h and then infected at an MOI of 0.1 with recombinant virus. (C and D) Viral titers at 24 h postinfection (hpi) of HFFs infected with HSV-1 variants in MEM with or without 0.1 mg/liter ferric nitrate [$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$]. Cells were treated with 100 IU/ml IFN- β (C) or vehicle control (D) for 18 h and then infected at an MOI of 0.1 with recombinant virus. The data points represent means $\pm$ SEM and are a summation of the results of three independent experiments. Statistical significance was determined by the two-tailed unpaired *t* test. (E) Western blots of HFFs with or without 0.1 mg/liter ferric nitrate and with 100 IU/ml IFN- β or vehicle control. Cells were treated for 24 h, and lysates were analyzed by immunoblotting using anti-phosphorylated STAT1 (P-STAT1), anti-STAT1, anti-p62, anti-LC3-I/II, and anti- β -actin antibodies. The immunoblots are representative of at least two independent experiments.

lanes 1 versus 2, 4 versus 5, 7 versus 8, and 10 versus 11). We did, however, observe a decrease in total Nrf2 in infection with $\Delta 68-87$ compared to strain 17 (Fig. 4A, lanes 1 and 2). Levels of antiapoptotic factor Bcl-xL and the apoptosis marker active caspase 3 were decreased upon infection with either $\Delta 68-87$ or strain 17, and this reduction was blocked by IFN- β pretreatment (Fig. 4A, lanes 1, 2, 7, and 8 versus lanes 4, 5, 10, and 11). We also quantified apoptosis and cell death by performing flow cytometry on annexin V- and propidium iodide-stained cells (Fig. 4B). Annexin V binds to phosphatidylserine which is present on the outer portion of the plasma membrane of cells undergoing apoptosis, while propidium iodide stains DNA and is not permeant to live cells. We did

FIG 4 Amino acids 68 to 87 of ICP34.5 do not alter global antioxidant response or apoptosis. (A) Western blots of HFF cells infected with HSV-1 strain 17 or Δ 68-87, or mock infected, at an MOI of 1 and harvested at 24 h postinfection (hpi). HFF cells cultured in MEM with or without 0.1 mg/liter ferric nitrate [$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$] were treated with 100 IU/ml IFN- β or vehicle control 18 h prior to infection. Lysates were analyzed by immunoblotting using anti-superoxide dismutase 2 (SOD2-Mn), anti-phosphorylated Nrf2 (P-Nrf2), anti-Nrf2, anti-Bcl-xL, anti-active caspase 3, anti-VP16 (viral infection marker), and anti- β -actin (loading control). The immunoblots are representative of the results of three independent experiments. (B) Mean fluorescence intensity (MFI) of annexin V (apoptosis marker) and propidium iodide (cell viability marker) staining by flow cytometry of HFFs infected with strain 17 or Δ 68-87 or mock infected. HFF cells cultured in MEM were treated with 100 IU/ml IFN- β or vehicle control 18 h prior to infection. At 12 hpi, cells were trypsinized, washed, stained with annexin V and propidium iodide, and analyzed by flow cytometry. Mean fluorescence intensity (MFI) values were normalized to their corresponding unstained controls. The data are representative of the results of three independent experiments.

not observe a difference in apoptosis between cells infected with Δ 68-87 or strain 17. As expected, cells treated with IFN- β showed elevated propidium iodide staining, but no difference was observed between Δ 68-87- and strain 17-infected cells. These findings indicated that although aa 68 to 87 are responsible for the interaction of ICP34.5 with multiple antioxidant and apoptosis factors, this region is dispensable for impacting the global responses of these pathways during infection.

ICP34.5 aa 68 to 87 alter mitochondrial motility and localization. The protein phosphatase PGAM5 regulates many aspects of mitochondrial dynamics, including mitochondrial trafficking, degradation, and biogenesis (49, 50, 68–73). Consequently, genetic ablation of PGAM5 in mice results in mitochondrion-related disorders and neurodegeneration (74–76). Additionally, HSV infection has been shown to alter mitochondria in neurons and keratinocytes (43, 44). We therefore hypothesized that interactions through the ICP34.5 domain from aa 68 to 87 serves to localize mitochondria to the site of virion production. This relocalization may be especially crucial in neurons due to their elongated morphology. We used virus infection of primary neurons to examine this hypothesis, based on the following observations: (i) ICP34.5 is a neurovirulence factor (54, 77–79), (ii) viruses lacking aa 68 to 87 of ICP34.5 are attenuated in neuronal tissues (27, 28, 57), and (iii) neurons are energetically demanding and rely heavily on targeted transport of mitochondria (40, 41, 80, 81). Using primary mouse superior cervical ganglion (SCG) neurons infected with mCherry-expressing HSV-1, we measured bulk mitochondrial velocity by time-lapse microscopy (Fig. 5A and B). We imaged live neuronal cultures stained with MitoTracker (pseudocolored teal) (Fig. 5A). In strain 17 mCherry-infected cells, mitochondria showed significantly reduced velocity compared to mock-infected cells, consistent with previous work (43). However, mito-

FIG 5 Amino acids 68 to 87 of ICP34.5 alter mitochondrial motility and localization. (A) Live-cell immunofluorescence images showing primary mouse superior cervical ganglion (SCG) cultures infected with HSV-1 strain 17 mCherry or Δ68-87 mCherry (red) and stained with MitoTracker (pseudocolored teal) to visualize mitochondria. The right panels show mitochondrial velocity over a 30-min period depicted by heat-mapped overlay with warmer colors representing faster mitochondria. (B) Blinded quantification of average velocity of mitochondria in SCG cultures infected with Δ68-87 mCherry or strain 17 mCherry or mock infected. SCG cultures were infected with HSV-1 variants at an MOI of 25 and, at 18 h postinfection (hpi), stained with MitoTracker and time-lapse imaged at a frequency of 2 min for 30 min. (C) Live-cell immunofluorescence images showing primary mouse trigeminal ganglion (TG) neuron cultures infected with HSV-1 strain 17 or Δ68-87 and stained with MitoTracker (pseudocolored teal) to visualize mitochondria. The right panels show mitochondrial velocity over a 3-min period depicted by heat-mapped overlay

(Continued on next page)

chondrial velocity was unaltered and near uninfected levels in cells infected with $\Delta 68-87$ mCherry. In addition, we measured axonal mitochondrial velocity in primary trigeminal (TG) neurons stained with MitoTracker (pseudocolored teal) (Fig. 5C; see Movies S1 and S2 in the supplemental material). Similar to the case for SCG neuronal cultures, average mitochondrial velocity in TG neuronal axons was significantly lower in strain 17-infected cells compared to $\Delta 68-87$ - or mock-infected cells (Fig. 5D). It has previously been shown that mitochondria in HSV-infected cells localize close to the outer rim of the nucleus (44, 45). Therefore, we wished to test whether perinuclear recruitment of mitochondria was due to ICP34.5 and dependent on its domain from aa 68 to 87. We stained infected SCG neurons for the mitochondrial marker TOMM20 and for HSV-1 using HA-tagged ICP34.5 as a marker of infection (Fig. 5E). The percentage of cells with perinuclear mitochondria was quantified by immunofluorescence microscopy (Fig. 5F). In strain 17-infected neurons, HA-positive cells displayed an abundance of mitochondria focused in the soma surrounding the nucleus (Fig. 5E, white arrowheads), while nearby HA-negative cells had normally distributed mitochondrial staining (Fig. 5E, yellow arrowheads). Compared to mock-infected cells, strain 17 infection resulted in significantly increased fraction of cells displaying dense perinuclear mitochondria (Fig. 5F). In contrast, $\Delta 68-87$ -HA infection did not provoke perinuclear clustering of mitochondria. In addition, we wished to address the possibility that differences in mitochondrial dynamics may be due to differences in viral replication between HSV-1 variants. We therefore quantified viral titers at 24 h postinfection (hpi) in SCG and TG neurons (Fig. 5G and H). In both types of neurons, replication of $\Delta 34.5$ and $\Delta 68-87$ was attenuated compared to that of strain 17 in IFN- β -pretreated cells. However, in the absence of IFN- β pretreatment, there was no difference in viral replication between the HSV-1 variants in either SCG or TG neurons. These results suggest that differences in mitochondrial motility and localization are not due to differences in viral replication. Together, these data demonstrate that ICP34.5 alters mitochondrial motility and localization through residues 68 to 87.

DISCUSSION

The HSV-1 protein ICP34.5 is a critical neurovirulence factor involved in multiple aspects of immune modulation. ICP34.5 blocks autophagy, facilitates virion nuclear egress, and reverses translational arrest, leading to maintained expression of viral factors involved in countering host defenses (23, 24, 26–28). The major contribution of ICP34.5 to virulence derives from its ability to dephosphorylate eIF2 α and reverse PKR-mediated translational arrest (82, 83). Previously, the blockade of autophagy by HSV-1 has been narrowed down to aa 68 to 87 of ICP34.5 (27). This domain is also important for neurovirulence, although deletion of this domain does not disrupt the ability of ICP34.5 to counter eIF2 α phosphorylation (27, 28). This study was designed to elucidate the mechanism behind the attenuation of viruses lacking aa 68 to 87 of ICP34.5. In an effort to remove bias, our approach relied on coimmunoprecipitation and tandem mass spectrometry experiments. This served to broaden the ICP34.5 protein interaction network and help reveal host pathways implicated during ICP34.5 immune

FIG 5 Legend (Continued)

with warmer colors representing faster mitochondria. (D) Blinded quantification of average velocity of mitochondria in axons of TG neurons infected with $\Delta 68-87$ or strain 17 or mock infected. TG cultures were infected with HSV-1 variants at an MOI of 25 and, at 18 hpi, stained with MitoTracker and time-lapse imaged at a frequency of 5 s for 3 min. (E) Immunofluorescence images of SCG neurons stained for mitochondrial marker TOMM20 (green), infection marker HA (red), neuronal marker β III-tubulin (pseudocolored teal), and nuclei (DAPI) (blue). SCG neurons were infected at an MOI of 25 and fixed at 20 h postinfection. In strain 17-infected cells, mitochondria concentrate near the nuclei (white arrowheads), while nearby uninfected cells show normally distributed mitochondria (yellow arrowheads). (F) Blinded quantification of the percentage of cells with perinuclear mitochondria in SCG cultures infected with $\Delta 68-87$ -HA or strain 17-HA or mock infected. The data points represent means $\pm$ SEM and are a summation of the results of three independent experiments. Statistical significance was determined by the two-tailed unpaired *t* test. (G and H) Viral titers measured at 24 hpi in SCG (G) and TG (H) neurons infected with HSV-1 variants. Cells were treated with 100 IU/ml IFN- β or vehicle control for 18 h and then infected at an MOI of 25 with recombinant virus. The data points represent means $\pm$ SEM and are a summation of the results of at least two independent experiments. Statistical significance was determined by the two-tailed unpaired *t* test.

modulation. Here, we built upon previous work (43, 44) and showed a direct association between altered mitochondrial dynamics and ICP34.5 function.

We provide support for previous work indicating that ICP34.5 binds C1QBP/p32 and PP1 α and that these interactions are independent of aa 68 to 87. The abundance of eIF2 α bound to the Δ 68-87 deletion protein was, however, less than that of eIF2 α bound to wild-type ICP34.5. This was unexpected since Δ 68-87 dephosphorylates eIF2 α during infection, and reversal of translational arrest by ICP34.5 is thereby independent of this domain (27, 28). The reason for this lower abundance is unclear but may reflect heretofore-unappreciated cooperative interactions between the multiple cellular proteins that bind ICP34.5. Our work also showed that ICP34.5 associated with numerous mitochondrial proteins, consistent with the idea that ICP34.5 localizes to mitochondria. ICP34.5 interacts and colocalizes with C1QBP/p32, a protein which decorates mitochondria (26). Our preliminary immunofluorescence and fractionation experiments, however, were inconclusive (data not shown), perhaps indicating transient or unstable mitochondrial localization. That said, our results show that ICP34.5 associates with multiple mitochondrial proteins, including PGAM5 and KEAP1, which provides insights into possible roles for ICP34.5 in the regulation of mitochondrial function.

Our replication data in DMEM recapitulate previous work showing only modest growth attenuation of Δ 68-87 *in vitro* in mouse and human cells (59, 60). Replication and neurovirulence of Δ 68-87, however, are far more attenuated *in vivo* than the *in vitro* data would predict (27, 28, 84). One explanation is that previous *in vitro* experiments were performed in either DMEM or complete neurobasal medium, which contain numerous redox buffers, including ferric nitrate (27, 58, 61). Here we have shown that ferric nitrate normalized the IFN-induced growth defect of Δ 68-87 in HFFs. Unfortunately, primary SCG and TG neurons do not survive in redox buffer-free MEM conditions, and therefore viral replication experiments under these conditions in primary neurons could not be performed. Given the interplay of ICP34.5 with multiple mitochondrial redox regulators, we hypothesize that the true replication defect of Δ 68-87 was masked by the presence of ferric nitrate. Iron is an essential component of a broad array of biochemical processes in the cell (85, 86). Together, these data provide evidence that modulation of redox and cellular metabolism is an important component by which ICP34.5 promotes virulence.

Localized to the cytoplasm and mitochondria, PGAM5 regulates programmed cell death pathways, the antioxidant response, and mitochondrial trafficking (49–53). Despite binding to PGAM5 being dependent on aa 68 to 87, activation of Nrf2 and levels of SOD2-Mn were unaltered in Δ 68-87 compared to wild-type virus. Furthermore, active caspase 3, Bcl-xL, and annexin V staining were all similar between Δ 68-87 and strain 17 infection, indicating no overall differences in apoptosis. PGAM5 also regulates cell death within the inflammasome pathway through a complex with RIPK1 and RIPK3 and promoting IL-1 β secretion (52, 87). However, we did not observe differences in overall cell death between Δ 68-87- and strain 17-infected cells. Furthermore, we did not detect RIPK1 or RIPK3 bound to ICP34.5 by coimmunoprecipitation-liquid chromatography (LC)-MS/MS analysis. There was, however, a clear phenotype for Δ 68-87 when we measured mitochondrial motility and localization. The phenotype was also present in HA-tagged ICP34.5 and the Δ 68-87 variant, which were generated independently to their untagged counterparts, suggesting that the observed phenotype is unlikely due to mutations outside the intended deletion of ICP34.5 aa 68 to 87. Altered mitochondrial dynamics are consistent with a demonstrated role for PGAM5 in the control of retrograde mitochondrial trafficking through a complex with KEAP1 and Nrf2 (50). This is of interest because infection with the alphaherpesviruses HSV-1, HSV-2, and PRV disrupts mitochondrial dynamics (43–45). While PRV lacks an ICP34.5 homologue, it alters intracellular calcium through a gB-dependent mechanism leading to mitochondrial dysfunction, highlighting the importance of this function during infection (43, 88). Although we have not elucidated a clear mechanism, we have demonstrated that aa 68 to 87 of ICP34.5 alter mitochondrial motility. Our lead hypothesis is that through interaction with PGAM5 and KEAP1, ICP34.5 causes mitochondrial clustering near the

nucleus, which is the site of virion production. The proximity of mitochondria and their associated antioxidant- and energy-producing activity to the nucleus is therefore important for relieving the bioenergetic demands on the cell during productive infection. Mitochondrial relocation is pivotal for provision of a local microenvironment for antioxidant activity and ATP synthesis (89, 90). Repositioning of mitochondria near the nuclei also facilitates transcription under oxidative stress (91). This could be especially important during acute infection of neurons, where distances are large and local energetic depletion might be problematic. In an HSV-2 genital model, others have shown that viral replication is decreased in mice lacking Nrf2 and that genetic activation of Nrf2 by deletion of its negative regulator, KEAP1, increases HSV-2 infectivity in mouse embryonic fibroblasts (92). Differences in viral replication could not account for changes in mitochondrial dynamics, since we observed no difference between $\Delta 68-87$ and wild-type HSV-1 replication in SCG or TG neurons in the absence of IFN- β pretreatment (Fig. 5G and H), and these data are consistent with previous work (59). Furthermore, the observed mitochondrial phenotype following HSV-1 infection may reflect dysfunctional motor proteins or their adaptors. For example, loss of the kinesin 1 motor protein results in accumulation of mitochondria near the nucleus at minus ends of microtubules (93–95). Disruption of components within the PGAM5/KEAP1/Nrf2 complex promotes Miro2-dependent retrograde trafficking of mitochondria (50). ICP34.5 may sequester one or more of these proteins and/or competitively inhibit their interactions. Additionally, mitophagy, an autophagic process of recycling mitochondria, occurs near the endoplasmic reticulum (ER) and involves beclin1 and PGAM5 (71, 96). A potential role of ICP34.5 in mitophagy is supported by previous work and therefore warrants further investigation (27).

In summary, we have provided a protein interactome for ICP34.5. We demonstrated that aa 68 to 87 of ICP34.5, which are important for neurovirulence, are necessary for binding PGAM5, KEAP1, and a number of proteins involved in mitochondrial dynamics. While this domain does not alter the total cellular antioxidant response or apoptosis, its redistribution of mitochondria likely sequesters energy and antioxidant factors to the site of viral production. The replicative attenuation of viruses lacking aa 68 to 87 of ICP34.5 was restored *in vitro* by the addition of ferric nitrate, an important cofactor in redox reactions and energy production. However, perturbation of mitochondria by HSV occurs regardless of medium condition (43–45). This suggests that the importance of redistributing mitochondria during viral replication is likely masked *in vitro* by artificial media rich in antioxidant molecules and bioenergetic cofactors. Rather than global changes to antioxidant gene expression, repositioning of antioxidant factors may be a strategy employed by HSV-1 to create a microenvironment which optimizes viral production. Taken together, these results link the HSV-related disruption of mitochondrial dynamics to ICP34.5. Removal of ICP34.5 from HSV-based oncolytic and gene delivery vectors attenuates the viruses (61, 97–101) and may also lower the possibility of neurodegeneration associated with mitochondrial disruption in neurons (75, 102–104).

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (HFFs), human embryonic kidney cells (HEK-293T), and African green monkey kidney (Vero) cells were from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone SH30022.01) or minimum essential medium (MEM) (Corning 10-010-CV) with 5% fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (HyClone) at 37°C and 5% CO₂. When noted, 0.1 mg/liter ferric nitrate [Fe(NO₃)₃·9H₂O] (Sigma-Aldrich) was dissolved in MEM to mimic the ferric nitrate content of DMEM. Primary superior cervical ganglion (SCG) neurons were isolated from 0- to 3-day-old C57BL/6J mice (Jackson Laboratory) as previously described (105) and were cultured on type I rat tail collagen (Corning)-coated glass in DMEM with 10% FBS, 1% penicillin-streptomycin, and 50 ng/ml nerve growth factor (NGF) (Gibco). Trigeminal ganglion (TG) neurons were isolated from 6- to 8-week-old C57BL/6J mice (Jackson Laboratory) as previously described (106). Mice were housed in the Center for Comparative Medicine and Research at the Geisel School of Medicine at Dartmouth, and procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All procedures were approved by the Dartmouth College IACUC. Wild-type strain 17, the $\gamma 34.5$ -null mutant ($\Delta 34.5$), and the strain 17 mutants lacking amino acids 87 to 106 (ΔXX ; here called

Δ 87-106), 68 to 106 (Δ BX; here called Δ 68-106), and 68 to 87 (Δ 68H; here called Δ 68-87) of ICP34.5 were made and described previously (27, 28, 54, 58, 107). To construct HA-tagged ICP34.5 viruses, a DNA fragment containing the PflMI-BstXI (bases 1073 to 1083 and 1686 to 1697; GenBank accession no. X14112.1 [108]) region of the γ 34.5 open reading frame containing an HA9 tag (TACCCATACGACGTCC CAGATTACGCG) at the C terminus was synthesized and cloned into pUC57 (GenScript, Piscataway, NJ, USA). The fragment was ligated into the previously described plasmids pDA04 and pDA07 to generate plasmids pDA04 34.5-HA and pDA07-HA (27). The resulting plasmids were linearized with XmnI and cotransfected into Vero cells with HSV-1 strain 17 genomic DNA using Lipofectamine reagent (Invitrogen). The resulting viruses were subjected to three rounds of plaque purification with screening by PCR and confirmation of mutation by DNA sequencing. To generate 17 mCherry and Δ 68-87 mCherry viruses, the mCherry open reading frame was cloned into the multiple-cloning site of plasmid pCI (Promega E1731). The BglII-BamHI region of the resulting plasmid, which contains mCherry under the CMV immediate early enhancer/promoter, was then cloned into the BglII site of plasmid pUIC-17 (strain 17 variant of pUIC) to produce plasmid pUIC-17 mCherry (109). The resulting plasmid was linearized and cotransfected into Vero cells with HSV-1 strain 17 or Δ 68-87 viral genomic DNA using Lipofectamine reagent (Invitrogen). Viral plaques were tested for successful homologous recombination by mCherry fluorescence. The resulting viruses were subjected to three rounds of plaque purification with screening by PCR and confirmation of mutation by DNA sequencing.

Plasmids and transfection. The plasmids p34.5-HA [p34.5(17)-HA], p Δ 68-106-HA [p Δ BX(17)-HA], p Δ 68-87-HA [p Δ BBD(17)-HA], and p Δ 87-106-HA [p Δ XX(17)-HA] and the empty vector control pCAGGS have been previously described (28, 110). Transfection of HEK-293T cells was performed using JetPRIME transfection reagent (Polyplus Transfection) at 1 μ g DNA per 10⁵ cells.

Virus infection. Cells were infected in either DMEM or MEM, as noted, containing 2% FBS for 1 h at 37°C with agitation every 15 min, followed by aspiration of inoculum and a brief wash with phosphate-buffered saline (PBS) (HyClone). Medium was replaced with DMEM containing 2% FBS or MEM containing 2% FBS with or without 0.1 mg/liter ferric nitrate as noted. SCG neurons were cultured for 4 days prior to infection to allow for extension of neurites and inoculated without agitation for 1 h at 37°C. Inoculum was aspirated, cells washed with PBS, and medium replaced with DMEM plus 10% FBS, 1% penicillin-streptomycin, and 50 ng/ml NGF. TG neurons were cultured for 4 days prior to infection to allow for extension of neurites and inoculated without agitation for 1 h at 37°C. Inoculum was aspirated, cells washed with Neurobasal-A medium (Gibco), and the medium was replaced with Neurobasal-A containing supplemental factors as described previously (106). Multiplicities of infection (MOI) of 0.1, 1, and 25 were used in experiments for Fig. 3, 4, and 5, respectively. Viral titers were quantified by standard plaque assay on Vero cells as previously described (111). When noted, cells were treated with 100 IU/ml recombinant human IFN- β (R&D Systems 8499-IF) or vehicle control (ethanol) 18 h prior to infection.

Immunoprecipitation and Western blot analysis. Cells were washed in PBS, harvested, and then lysed with 50 mM Tris pH 7.5 (Invitrogen), 150 mM NaCl (EMD Millipore), 1% Triton X-100 (Sigma), 0.5% sodium deoxycholate (Sigma), and 2 mM EDTA (BioWhittaker) for 1 h with agitation. Samples were spun down at 14,000 $\times$ g, and immunoprecipitation or Western blotting was performed on the supernatant. Immunoprecipitation was performed by incubating 1 μ g antibody per 20 μ l protein A/G-agarose beads (Santa Cruz Biotechnology sc-2003) for 1 h prior to incubation with cell lysate supernatants for 18 h at 4°C with nutation. Beads were washed for 10 min with each of the following buffers: (i) 15 mM Tris (pH 8), 150 mM NaCl, 4 mM EDTA, and 0.5% Triton X-100; (ii) 15 mM Tris (pH 8), 150 mM NaCl, 4 mM EDTA, and 0.5% Triton X-100; and (iii) 10 mM Tris (pH 8.0) and 1 mM EDTA. Cells and buffers were maintained at 4°C during sample preparation. Western blotting was performed by denaturing samples in 50 mM Tris-HCl (pH 6.8) (Invitrogen), 100 mM dithiothreitol (DTT) (Sigma), 2% SDS (Invitrogen), 0.1% bromophenol blue (Sigma), and 10% glycerol (National Diagnostics) at 95°C for 5 min. Polyacrylamide gel electrophoresis (PAGE) was performed, and either (i) gels were stained with a solution of 0.2% Coomassie blue R-250 (Sigma-Aldrich), 40% methanol, and 10% acetic acid and cleared with a 30% methanol–10% acetic acid solution to visualize total protein or (ii) proteins were transferred onto a polyvinylidene difluoride (PVDF) blotting membrane (EMD Millipore). The membrane was blocked with either 5% bovine serum albumin in Tris-buffered saline (TBS) with 0.1% polysorbate 20 (TBST) (Alfa Aesar) for phosphorylation-specific protein detection or 5% nonfat milk in PBS with 0.1% polysorbate 20 (PBST) for all others. Primary and secondary antibody incubations were done in blocking buffer for 18 h at 4°C and 1 h at room temperature, respectively. Primary antibodies used were rabbit anti-phospho-STAT1 (1:1,000; Cell Signaling Technology Y701), mouse anti-STAT1 (1:1,000; Santa Cruz Biotechnology M-23), rabbit anti-p62 (1:1,000; Novus NBP1-48320), rabbit anti-LC3 (1:1,000; MBL PD014), rabbit anti-PGAM5 (1:1,000; Abcam ab126534), rabbit anti-SOD2-Mn (1:1,000; Novus NB100-19925S), rabbit anti-phospho-Nrf2 serine 40 (1:15,000; Abcam ab76026), mouse anti-Nrf2 (1:1,000; Novus MAB3925), rabbit anti-Bcl-xL (1:1,000; Cell Signaling Technology 2764), rabbit anti-active caspase 3 (1:1,000; BD Biosciences 559565), mouse anti-HA.11 (1:5,000; BioLegend 16B12), rabbit anti-ICP34.5 (1:2,000; N69aa Patton [112]; kindly provided by Ian Mohr), rabbit anti- β -actin (1:5,000; BioLegend poly6221), and mouse anti-VP16 (1:5,000; Santa Cruz Biotechnology 1-21). Secondary antibodies used were goat anti-mouse–horseradish peroxidase (HRP) (1:25,000; Bio-Rad 170-6516) and goat anti-rabbit–HRP (1:25,000; Bio-Rad 170-6515).

LC-MS/MS. Prior to immunoprecipitation, mouse anti-HA.11 (BioLegend 16B12) antibodies were cross-linked to protein A/G-agarose beads with dimethyl pimelimidate (DMP) (Tokyo Chemical Industry) by methods previously described (113). Transfection, lysate preparation, and immunoprecipitation were as described above. Eluates were reduced with 5 mM DTT, alkylated with 15 mM iodoacetamide (Sigma-Aldrich), and precipitated with trichloroacetic acid (TCA) as described previously (114). Briefly, samples were precipitated in 20% TCA, washed three times in 10% TCA and three times in acetone

(stored at -20°C), and then vacuum centrifuged to remove any trace quantities of organic solvent. Precipitated proteins were digested to peptides with trypsin and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an EASY-nLC 1000 ultra-high-pressure liquid chromatograph (Thermo Fisher Scientific, Waltham, MA). Peptides were dissolved in loading buffer (5% methanol [Fisher]–1.5% formic acid [Sigma-Aldrich]) and injected directly onto an in-house pulled polymer-coated fritless fused silica analytical resolving column (40-cm length, $100\text{-}\mu\text{m}$ inner diameter; PolyMicro) packed with ReproSil C₁₈ AQ $1.9\text{-}\mu\text{m}$, $120\text{-}\text{\AA}$ pore (Dr. Maisch). Peptides in $3\text{-}\mu\text{l}$ loading buffer were loaded at 650×10^5 Pa of pressure by chasing onto the column with $10\text{-}\mu\text{l}$ loading buffer. Samples were separated with a 90-min gradient of 4% to 33% LC-MS buffer B (0.125% formic acid, 95% acetonitrile [ACN]) at a flow rate of 330 nl/min. The Orbitrap Fusion was operated with an Orbitrap MS1 scan at 120K resolution and an AGC target value of 500K. The maximum injection time was 100 ms, the scan range was 350 to 1500 m/z , and the dynamic exclusion window was 15 s (± 15 ppm from precursor ion m/z). Precursor ions were selected for MS2 using quadrupole isolation (0.7 m/z isolation width) in a “top-speed” (2-s duty cycle), data-dependent manner. MS2 scans were generated through higher-energy collision-induced dissociation (HCD) fragmentation (29% HCD energy) and Orbitrap analysis at 15K resolution. Ion charge states of +2 through +4 were selected for HCD MS2. The MS2 scan maximum injection time was 60 ms, and the AGC target value was 60K. Raw data were searched using COMET (115) against combined target-decoy versions of the human (*Homo sapiens*) and human herpesvirus 1 (HHV-1) (strain 17) proteome sequence databases (UniProt) with a precursor mass tolerance of ± 1.00 Da and requiring fully tryptic peptides with up to 3 missed cleavages, carbamidomethyl cysteine as a fixed modification, and oxidized methionine as a variable modification. The resulting peptide spectral matches were filtered to $\leq 1\%$ false-discovery rate (FDR) by defining thresholds of decoy hit frequencies at particular mass measurement accuracy (measured in parts per million from theoretical), Xcorr, and delta-Xcorr (dCn) values.

Flow cytometry. HFF cells were cultured in MEM with 5% FBS and 1% penicillin-streptomycin and IFN- β treated and infected as described above. Cells were trypsinized, washed with 2% FBS in PBS, and resuspended in PBS at 4°C . Apoptosis and cell viability staining were performed using the fluorescein isothiocyanate (FITC)-annexin V apoptosis detection kit I (BD Pharmingen) and analyzed on an Accuri C6 cytometer (BD Biosciences). In each treatment, mean fluorescence intensity (MFI) values were normalized to those for their corresponding unstained controls.

Immunofluorescence and live-cell imaging. Cells were fixed in 0.3% glutaraldehyde for 10 min, and unreacted aldehydes were reduced with three 10-min incubations in 1 mg/ml sodium borohydride (Alfa Aesar) in PBS, pH 8. Cells were permeabilized with 1% Triton X-100 and blocked with 4% normal goat serum (NGS) (Vector Laboratories) in 0.1% Triton X-100–PBS for 18 h at 4°C . Primary and secondary antibodies were diluted in PBS containing 2% NGS and 0.1% Triton X-100 and incubated on cells for 1 h at room temperature. Primary antibodies used were rabbit anti-TOMM20 (1:200; Abcam ab78547), mouse anti-HA.11 (1:1000; BioLegend 16B12), and chicken anti- β III-tubulin (1:500; Millipore-Sigma AB9354). The DNA stain 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen 62248) was used to stain nuclei. Secondary antibodies used were goat anti-mouse Alexa 555, goat anti-rabbit Alexa 488, and goat anti-chicken Alexa 647 (1:500; Invitrogen). For live-cell imaging, cells were stained with 15 nM Mito-Tracker Deep Red FM (Invitrogen M22426) in phenol red-free DMEM for SCG neurons or supplemented Neurobasal-A (106) for TG neurons for 15 min at 37°C . Excess dye was washed, medium replaced with complete DMEM or Neurobasal-A containing 20 mM HEPES, and cells imaged on a 37°C heated stage. Images were collected every 2 min for 30 min per field for SCG neurons and every 5 s for 3 min per field for TG neurons. All imaging was performed using a Zeiss Axio Observer.Z1 with a $20\times$ Plan-ApoChromat 0.8 NA air (Zeiss) and a $63\times$ Plan-ApoChromat 1.4 NA oil (Zeiss) objective and Zeiss Zen Blue software. For analysis blinding, samples for fixed and live-cell imaging were randomized immediately following viral infection and decoded after all replicate experiments had been performed and analyzed. Perinuclear mitochondria were counted manually, and nuclei were quantified using ImageJ (116). Bulk mitochondrial velocity analysis in SCG cultures and velocity heat-mapped overlays were performed using ImageJ, the ImageJ distribution, Fiji, and the plugin TrackMate (116–118). Axonal mitochondrial velocity in TG neurons was blinded and quantified manually by tracking at least three mitochondria per time-lapse field among two technical replicate samples per three independent experiments. Unless otherwise stated, all statistical analysis and graphical representations were generated using Prism 8 (GraphPad).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.4 MB.

SUPPLEMENTAL FILE 2, AVI file, 4.2 MB.

SUPPLEMENTAL FILE 3, AVI file, 2.2 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

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