Viral factors of poxviruses and coronaviruses that suppress host gene expression

by

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B.S., Kansas State University, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Host shutoff is the phenomenon observed when many viruses downregulate host cellular gene expression resulting in a global suppression of nascent protein production. A virus can encode multiple factors that interfere at different stages of gene expression from transcription, mRNA processing and export, to translation. While much work has been done to define those viral factors that induce host shutoff, more needs to be done to fully characterize those host shutoff factors and identify novel host shutoff factors that could potentially elucidate previously unknown mechanisms underlying viral-host interactions. The development of innovative techniques will improve our ability to elucidate novel mechanisms involved in viral host shutoff.

The work in this dissertation addresses this by developing of a novel high throughput assay that allows for rapid, quantitative, and consecutive monitoring of host shutoff and also studying viral host shutoff factors and screening for previously unknown host shutoff factors. Our results demonstrated high-throughput assay utilizing a cell line stably expressing *Gaussia* luciferase could be used to analyze host shutoff from a single VACV infected sample quantitatively and in real time. Utilizing the Gaussia assay, our results showed the strongest inhibition of protein synthesis during VACV infection came from post-replication of the viral genome. We also showed VACV was able to induce host shutoff while encoding inactive/deleted decapping enzymes, suggesting other late gene viral factors may contribute to host shutoff. Next, we developed a VACV late gene screening assay to identify additional viral late genes that contributed to host shutoff. The results from this screening identified eight late genes that were possibly capable of inducing host shutoff, a function not previously defined for these genes.

Coronavirus disease 2019 is a respiratory illness caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Multiple vaccines and therapies have been developed to mitigate the risk of this disease to the public, but a better understanding of the virus and its pathogenesis will allow for improved treatments for the current and future coronavirus pandemics. The work in this dissertation addressed this by characterizing the host shutoff factor nsp1 of SARS-CoV-2 and performed a screening to find other viral genes of SARS-CoV-2 that contribute to host shutoff. Our results show nsp1 is capable of inducing host shutoff, and residues R124 and K125 in the N-terminal, residues K164 H165 in the C-terminal, and residues 122-130 are critical for nsp1 SARS-CoV-2 host shutoff function. We then screened all the SARS-CoV-2 viral genes for their ability to induce host shutoff utilizing our EF1a-*Gaussia* luciferase reporter plasmid and showed that some structural and accessory proteins were potentially capable of inducing host shutoff as well as nsp5, the SARS-CoV-2 viral proteinase.

Taken together, this work develops new tools that can be used to study protein synthesis suppression by viral infection and other physiologically relevant stresses, as well as identifies new viral host shutoff factors; both of which contribute to the current understanding of virus-host interactions.

Viral factors that suppress host cellular translation

by

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Dedication

This work is dedicated to my parents. Your love has had a tremendous influence on my life.

Chapter 1 - Introduction

Virus-Host Interaction

Viruses are obligate intracellular parasites that rely on host processes for replication. Viruses depend on host translation machinery for the synthesis of viral proteins. The process of translation is very energy consuming, and the viral mRNA has to compete with cellular mRNA. Most viruses have developed mechanisms to decrease the synthesis of host proteins and prioritize viral protein synthesis, a process termed host shutoff. This not only directs the cells energy and amino acids to synthesis of viral proteins, but also can inhibit the cell immune response. While some viral factors are well known, many factors that contribute to host shutoff have not been well characterized. New research into host shutoff will give one new insight into virus-host interactions which can lead to vaccine and therapeutic developments, along with improved oncolytic agents. The study of viruses will continue to advance the fields of cellular biology and immunology, and many more.

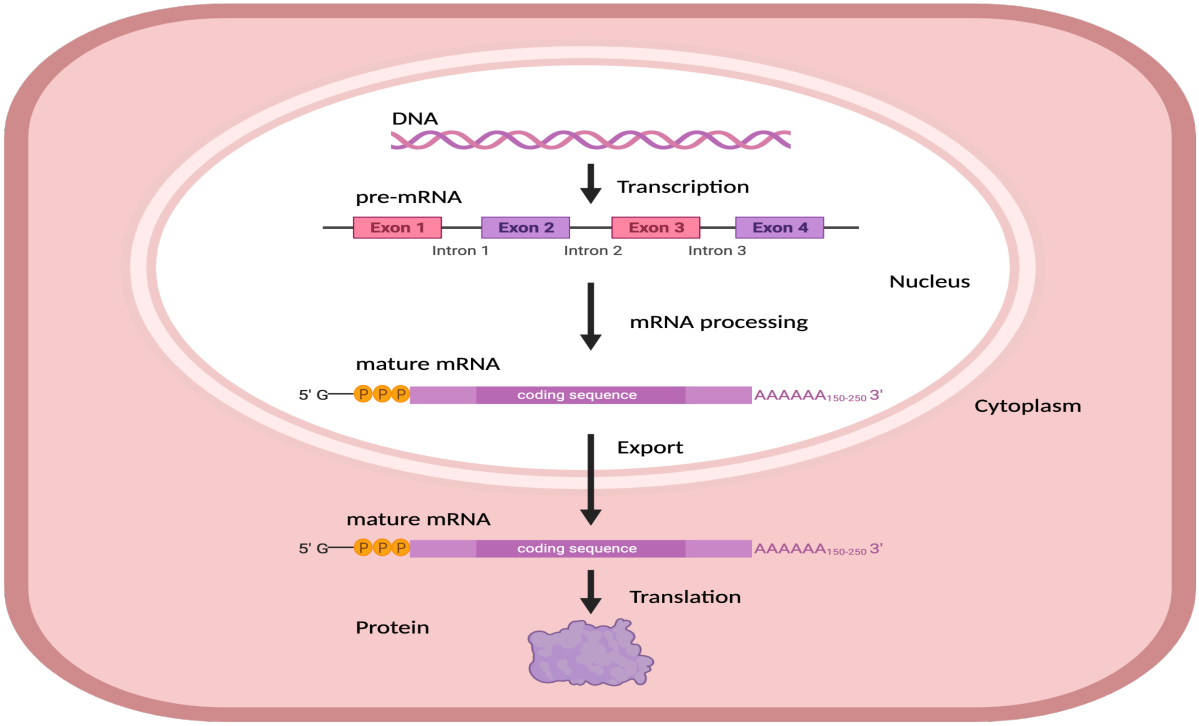
Viruses may induce host shutoff at different levels of gene expression from transcription, mRNA maturation and transport, to translation. These processes will be briefly covered here and show how they are interconnected with one another.

The Pathway From DNA to Protein

Cells store their hereditary or genetic information in the form of nucleotide sequences in a deoxyribonucleic acid (DNA) molecule called a gene. These cellular genes are used as a template to synthesize the functional units of the cell in the form of a proteins or RNA. Genes

cannot synthesize proteins directly but use an RNA molecule termed messenger RNA (mRNA) as an intermediary that is made through a process called transcription using the DNA sequence as a template. The mRNA is then used as a template for the synthesis of proteins in a process called translation (see Figure 1.1). This expression of cellular genes occurs by the same mechanism in prokaryote and eukaryotic cells and is known as the central dogma of molecular biology. While the fundamental processes are the same between prokaryote and eukaryote cells, variations do exist such as mRNA processing, and for the rest of this introduction, gene expression in the context of eukaryotic cells will be discussed. Many factors are involved in the processes that make up the central dogma, I will briefly introduce transcription, mRNA processing and export, and translation as they are well studied targets of virus host shutoff mechanisms.

Figure 1.1



The Pathway From DNA to Protein. The expression of cellular genes is known as the central dogma of molecular biology. The process involves several mechanisms including transcription, mRNA processing and export, as well as translation. Created with BioRender (2022).

Transcription

Transcription is the process of synthesizing single stranded RNA by sequential addition of nucleotides that are complementary DNA template by the enzyme RNA polymerase. As mentioned previously, a gene whose functional unit is a protein will be transcribed into mRNA by RNA polymerase II (RNAPII) and is considered a coding RNA. Some RNAs serve as enzymatic, structural, and regulatory components in many cellular processes, termed non-coding RNAs, and are transcribed by RNA polymerase I and III. As my focus is host shutoff, I will focus on coding RNAs and RNAPII. Transcription by RNAPII can be divided into four distinct stages: recruitment, initiation, elongation, and termination. RNAPII is recruited to gene promoter by the transcription initiation complex. The transcription initiation complex is made up of general transcription factors that recognize the promoter sequence, recruit, and position of RNAPII at the transcription start site, and releases RNAPII from the promoter. RNAPII then undergoes what is called promoter-proximal pause (PPP), and elongation factors are required to release RNAPII from the PPP and help stabilize RNAPII to allow transcription of the downstream gene. RNAPII will continue to transcribe the DNA until it reaches a termination stop point, where it will release the nascent RNA and eventually disassociate from the DNA template. Transcription termination is facilitated by termination factors and prevents the expression of adjacent genes (Alberts et al., 2015).

mRNA Processing

The nascent pre-mRNA synthesized during transcription must undergo three major processing events to be considered mature mRNA which will be exported out of the nucleus and translated into a polypeptide. The three processing events include the addition of a 5' end modified guanine nucleotide cap, splicing long intervening non-coding sequences called introns out of the mRNA body, and generation of a 3' end polyadenylation tail (poly(A) tail). While mRNA processing was thought to occur post-transcription, and is often how it is depicted in figures, these modifications occur co-transcriptionally and are coupled with other parts of RNA metabolism through the flexible carboxyl-terminal domain (CTD) of RNAPII. CTD is involved in associating different factors involved with transcription and RNA processing to RNAPII. The addition of the 5' cap occurs early during translation, and helps the cell recognize mRNA, as RNAPI and III lack the flexible CTD, and their nascent noncoding RNA do not undergo the same modifications. The 5' cap binds to the cap binding complex and aids in further processing of the mRNA, as well as nuclear export and eventually binds to translation factors to aid in translation. Eukaryotic pre-mRNA does not contain a continuous coding region like prokaryotes, but has long noncoding sequences called introns that interrupt the expressed coding regions called exons. The introns must be spliced out before the mRNA can be exported and efficiently translated. The splicing of pre-mRNA occurs co-transcriptionally after the addition of the 5' cap. Genes can code for multiple protein products through a process called alternative splicing that can splice the pre-mRNA different ways. The position of the 3' end poly(A) tail is encoded within the genome and is recognized by processing factors that recognize and bind to the site as it is transcribed and triggers cleavage of the nascent RNA by RNAPII. After cleavage of the

nascent RNA, a poly(A) polymerase will add a string of adenines. After processing, the mature mRNA will be exported out of the nucleus into the cytosol where it will be translated into a polypeptide (Alberts et al., 2015).

Translation

Protein synthesis, or translation, occurs outside the nucleus in the 80S eukaryotic ribosome which is made of the 60S (large) and the 40S (small) subunits. The 40S small subunit is recruited to mRNA as part of the 43S translation initiation complex made up of 40S small ribosomal subunit and several eukaryotic translation initiation factors, eIFs. eIF4F is responsible for binding to the 5' cap structure of the mRNA, while eIF4A is the ATP-dependent RNA helicase that unwinds the secondary structure of the mRNA, and eIF4G that acts as a scaffolding protein also interacts with the poly(A) binding protein promoting circularization of the mRNA. Once 40S small ribosomal subunit is recruited to the 5' cap it starts to scan the mRNA until it recognizes the initiation codon through interaction with the initiator transfer RNA (tRNA). After recognition of the start codon the 60S large subunit is recruited to the translation complex and this process initiates translation. The ribosome reads the mRNA three nucleic acids at times called a codon, and this codon corresponds to a specific amino acid carried by a tRNA. As the ribosome reads the mRNA, amino acids are continuously added to the growing polypeptide chain with the help of elongation factors. One single mRNA molecule of multiple ribosomes can synthesize polypeptides simultaneously; this is termed a polysome. Translation release factors recognize the stop codon causing the translational complex to dissociate and the polypeptide is released. Ribosomes can synthesize proteins within the cytosol as free ribosomes, or on the

rough endoplasmic reticulum by membrane-bound ribosomes. Proteins translated on the rough endoplasmic reticulum include transmembrane proteins that have one or more hydrophobic regions that are inserted into the membrane or proteins carrying a signal peptide that will be trafficked through the secretory pathway for secretion, or to another destination such as the endoplasmic reticulum, Golgi, lysosome, or plasma membrane. Multiple types of posttranslational modifications occur within the endoplasmic reticulum and the Golgi (Alberts et al., 2015).

Suppression of Gene Expression by Notable Viruses

The gene expression regulation by virus infection occurs a number of ways, such as degradation of cellular mRNAs, alteration of the activity of ribosome and associated factors, competitive displacement of cellular mRNAs by viral mRNAs for translation, and many more. Here I will cover a few notable viruses that have multiple viral factors that lead to host shutoff and discuss important factors that may be considered for future investigations into viral induced host shutoff (see Table 1.1).

Table 1.1

Host Shutoff Factors of Notable Viruses and Their Targeted Cellular Processes

Host	Viral factors	Targeted cellular processes
HSV	CHPK (MCMV)	Cellular DNA replication
	ICP4	Transcription initiation
	ICP22	Transcription elongation
	ICP27	Transcription termination; mRNA splicing
	VHS	Accelerated mRNA degradation
IAV	RdRp-PA	Cap snatching

	NS1	mRNA processing/nuclear export
	PA-X	Accelerated mRNA degradation
VACV	K7	Histone methylation
	H1	mRNA splicing
	D9	mRNA decapping
	D10	mRNA decapping
	F17	Translation initiation
	VACVWR169	Translation
	VACV-poly(A)-polymerase	Translation
CoV	Nsp1	mRNA degradation, ribosome binding, mRNA export
	ORF6	mRNA export
	NSP14-NSP10	mRNA processing
	NSP16-NSP10	mRNA splicing
HIV	Vpr	mRNA splicing
	PR	Translation initiation
	Gag	Translation initiation

HSV

Herpesviridae is a family of linear DNA viruses that can go through lytic and latent infections within the host. Herpesvirus can be divided into three subfamilies—alpha, beta, and gamma. Herpes simplex virus 1 belongs to alpha-viridae and has been shown to induce host shutoff during the lytic phase of infection when the virus is reactivated with a 70% reduction in protein synthesis in the first 3h (Hennig et al., 2018; Roizman & Roane, 1964). HSV-1 genome replication, transcription, and genome packaging all occur within the nucleus, and HSV-1 does not encode a viral RNA polymerase, so it must compete with cellular gene for RNAPII transcription (Packard & Dembowski, 2021). HSV-1 utilizes several mechanisms to inhibit different stages of transcription to selectively downregulate cellular gene transcription and upregulate transcription of viral genes.

Transcription. Alterations in transcription processes during HSV infection results in a ~50% reduction in RNA synthesis (Roizmann & Roane, 1964). A major contributor to alterations in RNA metabolism is targeting of RNAPII by HSV-1 to disrupt all four phases of transcription: recruitment, initiation, elongation, and termination. But while specific targeting RNAPII by viral protein has been shown, reduced activity of multiple RNA polymerases has been observed (Preston & Newton, 1976; Spencer et al., 1997). The decreased activity of RNA polymerases during HSV infection could not be completely attributed to the reduction of cellular protein synthesis, suggesting factors other than RNAPII targeting, and host shutoff may be involved (Preston & Newton, 1976). Here I will briefly summarize a few viral factors that regulate transcription and contribute to host shutoff. Hennig et al. (2018) has a more in depth look into HSV alterations of cellular transcription.

In addition to a decrease in cellular RNA synthesis, cellular DNA synthesis is reduced after HSV infection (Roizman & Roane, 1964). Recently it was shown that the conserved herpesvirus protein kinase (CHPK) M97 of mouse cytomegalovirus (mouse β-herpesvirus) sequesters Cyclin A in the cytosol and this interaction is required for viral inhibition of cellular DNA replication (Bogdanow et al., 2020). The CHPKs are included on the list core genes that are conserved in all herpesviruses, and it would be interesting to determine if they contribute to host shutoff. Even though β-herpesviruses induce host transcription shutoff, they do not induce a global host shutoff of protein synthesis as discrepancies between mRNA abundance and protein expression have been identified (Stanton et al., 2007). This discrepancy demonstrates the complexity of regulation of cellular processes and suggests multiple factors are involved in the regulation of synthesis of cellular proteins such as mRNA stability, and β-herpesviruses are more

selective in modulation of gene expression (Hertel & Mocarski, 2004; Stanton et al., 2007; Zhu et al., 1998). This is supported by the fact that RNAPII levels are reduced on a majority of genes but is actively recruited to select genes (Birkenheuer et al., 2018). A reduction in cellular DNA synthesis would result in less transcripts for RNA polymerases, and potentially a decrease in cellular RNA synthesis and thus contribute to a reduction in cellular gene expression.

Infected cell polypeptide 4 (ICP4) has been shown to reduce RNAPII occupancy on cellular mRNA promoters and increase RNAPII occupancy on viral genomes and is involved in the temporal regulation of viral gene expression (Courtney & Benyesh-Melnick, 1974; T. Rivas et al., 2021). ICP4 has also been shown to interact with cellular transcription factors such as transcription factor II D, a component of the RNAPII preinitiation complex, resulting in the activation viral transcription (Dembowski & DeLuca, 2015; L. M. Wagner & DeLuca, 2013). Dremel and DeLuca (2019) also found ICP4 selectively bound to cellular genes that are involved in common housekeeping functions. Thus, ICP4 contributes to global reduction in cellular RNA synthesis and activates transcription of certain cellular genes to promote viral replication.

The HSV-1 immediate-early protein ICP22 contributes to inhibition of cellular gene expression and promotes viral gene expression through regulation of transcription elongation (Rice & Davido, 2013). ICP22 is recruited to cellular genes where it interacts with the positive transcription elongation factor b resulting in a decrease phosphorylation of Ser2 of the CTD RNAPII by CDK9 (Birkenheuer & Baines, 2020; Isa et al., 2021; Zaborowska et al., 2014). CDK9 plays a role in transcription elongation by phosphorylating RNAPII at serine 2 of the CTD thus triggering the release of RNAPII from the PPP site allowing transcription of the

downstream gene (Carey et al., 2008). While HSV-1 targeting of RNAPII results in a global inhibitory effect on transcription as seen with RNAPII occupancy on a majority of genes is decreased, it has been demonstrated that RNAPII occupancy on select genes are increased and ICP22 may stimulate selective transcription of cellular genes by stabilizing RNAPII at important nascent RNA whose expression enhances HSV-1 replication (Birkenheuer et al., 2018; Birkenheuer et al., 2022). ICP22 has been shown to enhance viral gene expression and Pro-Seq data has shown ICP22 contributes to RNAPII stability at the PPP site of viral genes (Birkenheurer et al., 2022). ICP4 has been shown to promote release of RNAPII at these PPP sites (T. Rivas et al., 2021). This dynamic regulation of RNAPII may allow efficient transition from early viral gene expression to activation of late gene expression.

HSV-1 infection has been shown to disrupt transcription termination of cellular genes and lead to an increase in chromatin accessibility downstream of individual genes all of which result in read-in transcription (Hennig et al., 2018). Of the 10,000 cellular genes studied, 53% showed >35% readout, and this led to a greater reduction in translation rates of these genes. The aberrant transcripts resulting from disruption in transcription termination may interfere with splicing of the mRNA, nuclear export, or translation (Rutkowski et al., 2015). The mechanism behind disruption of transcription termination has not been fully elucidated, but recent evidence suggests the disruption of mRNA 3' processing of host genes by ICP27 blocks transcription termination (Wang et al., 2020). Other studies have suggested ICP27 was not required for disruption of transcription termination (Rutkowski et al., 2015). Further studies elucidating the mechanisms of viral regulation of host transcription will reveal additional factors that contribute to host shutoff.

mRNA Processing and Trafficking. HSV-1 mutants lacking ICP27 reduced the ability of the virus to induce host shutoff (Sacks et al., 1985). ICP27 has been shown to reduce the amount of spliced cellular mRNAs causing an accumulation of pre-mRNA in the nucleus (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994). As the HSV viral genes are intronless, with a few exceptions, targeting of the cellular splicing machinery would contribute to host shutoff (He et al., 2021). ICP27 protein is also involved in stimulating viral gene transcription, efficient export of viral mRNA, initiating translation of viral mRNA, and inhibition of interferon signaling (Sandri-Goldin, 2011). Recent evidence has shown that ICP27 block of cellular mRNA splicing may not be direct, but disruption in splicing may be due to aberrant mRNA from a disruption in transcription termination by ICP27 inhibition of 3' mRNA processing (Rutkowski et al., 2015; Wang et al., 2020). ICP27 demonstrates the significance of the interconnectedness of cellular processes, and how disruption of many cellular mechanisms can contribute to virus induced host shutoff allowing the cell to inhibit the innate immune response allowing for efficient viral replication.

The HSV-1 UL41 gene encodes for the virion host shutoff (VHS) nuclease protein that degrades cellular and viral mRNA facilitating a decrease in cellular protein synthesis and the temporal regulation of viral mRNA (Hardwicke & Sandri-Goldin, 1994). VHS selectively degrades mRNA though interaction with translation initiation factors, and this accelerated turnover of mRNA leads to global host shutoff (Everly et al., 2002). VHS is also responsible for limiting the accumulation of dsRNA, thus blocking the activation of protein kinase R (Dauber et al., 2019; Sciortino et al., 2013). The gamma-herpesvirus Kaposi sarcoma-associated herpesvirus

and Epstein-Barr virus encode SOX and BGL5 respectively, and these proteins accelerate mRNA degradation and induce host shutoff (Y. J. Lee & Glaunsinger, 2009; Rowe et al., 2007).

Translation. HSV-1 contains viral factors that interact with translation machinery and stimulate translation of viral transcripts. Walsh and Mohr (2011) published a comprehensive review of the HSV and other viral factors that interact with translation machinery (Walsh and Mohr, 2011). While HSV-1 has viral proteins that target translation machinery and signaling pathways such as PKR and UPR, none of these are a result of host shutoff with the exception of VHS (Glaunsinger, 2015). Further research may elucidate new mechanisms of translation regulation and uncover new viral factors that contribute to host shutoff.

IAV

Influenza virus (IAV) is a negative strand RNA virus that replicates in the nucleus of host cells and is known to cause global inhibition of cellular protein synthesis (Inglis, 1982). The main mechanisms of IAV induced host shutoff are blocking of mRNA processing, degradation of RNAPII, and accelerated degradation of mRNA (H. G. Rivas et al., 2016). While some viruses have mechanisms to translate viral mRNAs more efficiently than cellular mRNAs, it has been shown that translation efficiency of viral and cellular mRNAs during IAV infection is similar, thus the reduction of the concentration of cytoplasmic cellular mRNAs is thought to be the reason for host shutoff (Burgess & Mohr, 2015). Each of the mechanisms used by IAV would reduce the concentration of cellular mRNAs and lead to reduced cellular protein synthesis (Inglis, 1982). Thus, IAV induced shutoff demonstrates how a virus can regulate multiple

molecular mechanisms to reduce global protein synthesis. H. G. Rivas et al. (2016) provides a more in-depth review on IAV host shutoff.

Transcription. IAV encodes a viral RNA-dependent RNA-polymerase (RdRp) to transcribe viral mRNAs. The synthesis of viral mRNAs by IAV requires a mechanism called cap snatching, where RdRp and other viral factors such as the polymerase acidic (PA) protein associate with cellular RNAPII and cleave the 5' capped-end of cellular mRNA along with 10-15 nucleotides that acts as a primer for viral mRNA transcription (Bouloy et al., 1979; Chan et al., 2006; Plotch et al., 1979). The association of RdRp-PA with cellular RNAPII results in reduced RNAPII elongation and triggers cellular RNAPII degradation (Chan et al., 2006; Vreede et al., 2010). This has been confirmed with the observation of reduced cellular RNAPII occupancy in gene bodies relative to the transcription start site (Zhao et al., 2018). Because IAV encodes its own viral RNA polymerase to transcribe viral mRNAs, alterations to the function of cellular RNAPII would selectively down regulate the transcription of cellular mRNAs while not inhibiting viral mRNA synthesis.

mRNA Processing and Trafficking. Along with decreased RNAPII occupancy of gene bodies downstream of the transcription start site, it was also observed that IAV infection disrupts the termination step of transcription which leads to read-in transcription, a similar effect caused by HSV-1 resulting in aberrant transcripts that have a reduced translation efficiency (Zhao et al., 2018). The disruption in transcription termination by IAV is caused by NS1, a multifunction viral protein that contains two previously characterized domains, the N-terminal RNA binding domain and the C-terminal effector domain (Qian et al., 1994). The C-terminal RNA binding

domain of NS1 has been shown to disrupt host immune response by binding to dsRNA and inhibiting the activation of the OAS/RNase L pathway (Das et al., 2008). The effector domain is responsible for binding to and blocking the function of cleavage and polyadenylation specificity factor (CPSF), an essential component of the pre-mRNA 3'-end processing machinery (Nemeroff et al., 1998). This prevents the cleavage of the 3'-end and addition of the poly(A) tail to pre-mRNA which leads to read-in transcription as well as dysregulating mRNA processing events that affect translation efficiency such as mRNA splicing and export from the nucleus (Davidson et al., 2014). Viral pre-mRNA processing remains unaffected by this mechanism of inhibition as their 3' poly(A) tail is synthesized by RdRp slippage (Poon et al., 1999). This leads to the selective nuclear export of viral mRNAs as cellular mRNA nuclear export is blocked thus leading to host shutoff (Chen & Krug, 2000). Despite the global reduction in cellular mRNA processing caused by read-in transcription, some genes are upregulated, suggesting their pre-mRNA may be processed differently than other cellular mRNA. This reinforces the idea that some cellular genes are efficiently translated during viral induced host-shutoff (Zhao et al., 2018).

The viral endoribonuclease PA-X contributes to host shutoff by selectively degrading host mRNAs in the nucleus (Khaperskyy et al., 2016). PA-X protein synthesis is a result of a ribosomal frameshift during translation of the viral PA protein, and while it still contains the endonuclease domain of PA, but its C-terminal region is encoded by the second reading frame labelled X-ORF, which lacks the binding domain that interacts with RdRp (Firth et al., 2012; Jagger et al., 2012). Khaperskyy et al. (2016) showed the C-terminal X-ORF is required for PA-

X activity, and PA-X selectively degrades RNAs transcribed by RNAPII (Khaperskyy et al., 2016). PA-X is able to selectively degrade host mRNA by interacting with proteins involved with cellular RNA metabolism, which allows viral mRNAs that are processed non-canonically to escape this host shutoff mechanism. This is supported by the finding that PA-X selectively targets spliced transcripts over intronless RNAs (Gaucherand et al., 2019). While PA-X induces a global downregulation of host RNAs, some RNAs involved in cellular processes such as transcription, translation, rRNA processing, ribosomal proteins, and others were PA-X resistant.

VACV

Vaccinia virus is a member of *Poxviridae*, a family of large double-stranded DNA viruses that replicate entirely in the cytoplasm. Poxviruses are divided into two subfamilies based on vertebrate and insect host range, termed Chordopoxvirinae and Entomopoxvirinae respectively (Moss, 2013). Poxviruses can cause many diseases; most notably is smallpox caused by Variola virus which is responsible for many millions of deaths according to the World Health Organization and in 1980 the disease was declared eradicated through a global vaccination program (Fenner, 1993). Poxviruses that infect both animals and humans such as ORF virus, cowpox, and monkeypox can cause human disease, while other poxviruses such as Capripoxvirus can infect cattle, sheep, and goat and can result in devastating economic losses (Yang et al., 2021). VACV is the prototypic orthopoxvirus and was used as the vaccine to eradicate smallpox.

VACV has been shown to induce host shutoff upon infection and can start as early as 20 minutes post infection, suggesting the virion may contain factors that contribute to host shutoff

(Moss, 1968). Several strategies used by VACV to induce host shutoff have been characterized. VACV can disrupt protein synthesis by targeting the transcription, mRNA processing, and translation processes. These have been reviewed in detail previously (Dhungel et al., 2020), but we will focus on how VACV has multiple strategies to induce shutoff.

Transcription. While many viruses target RNAPII enzymes to inhibit transcription, the mechanism by which VACV interferes with host transcription has yet to be fully elucidated. Experiments utilizing radioactive prelabeled thymidine-H3 or 3H-uridine have shown that while the DNA of the host cell is not degraded, there is a rapid inhibition of DNA and RNA synthesis (Kit & Dubbs, 1962; Pedley & Cooper, 1984). Reduced RNA synthesis would result in a decrease in the host mRNA pool directly affecting host protein synthesis. Whether or not inhibition of cellular DNA synthesis contributes to host shutoff is unclear. Recently it has been shown the VACV infection results in increased histone methylation that is characteristic of chromatin repressed complexes. Teferi et al. (2017) determined VACV K7 gene was the main contributor but did not rule out other viral factors affecting histone methylation may be involved. This finding suggests epigenetic modifications may contribute to the decrease in RNA synthesis. Interestingly, K7 has also been shown to reduce phosphorylation of IKKα, a key part of the interferon induction pathway, by targeting dead box protein 3 RNA helicase. A similar mechanism was found to be utilized by the murine cytomegalovirus m139 protein (Puhach et al., 2020; Schröder, 2010; Schröder et al., 2008). This is one example demonstrating how one viral protein can have multiple functions, and how different viruses can share common strategies to interact with host pathways to enhance viral replication.

mRNA Processing. VACV mRNA have been shown to be intronless thus inhibition of host cell mRNA splicing machinery by VACV would selectively decrease the amount of mature host mRNA while not regulating viral mRNA (Wittek et al., 1980). VACV targets host cell RNA splicing machinery function by inactivating SR proteins. The host factor involved in the dephosphorylation of SR proteins has yet to be elucidated. A good candidate would be the H1 VACV phosphatase protein as it has been shown to be capable of dephosphorylating SR proteins but has not been shown in the context of viral infection (Huang et al., 2002). Adenovirus has also been shown to inactivate SR proteins in infected cells. When Adenovirus inactivated SR proteins were incubated in conditions that reversed their phosphorylation status, their enhancing and suppressor activity was restored completely, while VACV inactivated SR proteins were only partially restored (Huang et al., 2002). This would suggest these viruses use different mechanisms to target SR proteins and regulate their activity.

Rapid mRNA turnover resulting in down regulation of gene expression in VACV infected cells is induced by VACV nudix hydrolase decapping enzymes D9 and D10 (Parrish & Moss, 2007; Parrish et al., 2007). Evidence has shown that both proteins regulate mRNA in a cap dependent manner (Shors et al., 1999). Both viral genes are highly conserved in most chordopoxviruses, D10 being conserved in entomopoxviruses as well (Parrish et al., 2007). The decapping of mRNAs by D9 and D10 makes them mRNA susceptible to degradation by the host exonuclease Xrn1, and D9 and D10 have been shown to prevent accumulation of dsRNA blocking host anti-viral responses (Burgess & Mohr, 2015; S-W. Liu et al., 2015). The conservation of two decapping enzymes by chordopoxvirus may be due the temporal regulation of D9 and D10, with D9 expressed as an early gene, and D10 being expressed after DNA

replication (Parrish et al., 2007). It may also be due to different mRNA targets, as both genes contain the nudix hydrolase motif, but only share 25% sequence identity. The flanking regions of these decapping enzymes is under investigation and are thought to regulate protein function and mRNA targeting.

Translation. The VACV virion was shown to induce host shutoff by inhibiting the formation of the methionyl-tRNA_fMet-40S initiation complex in infected cells treated with viral gene expression inhibitor (Person et al., 1980). It was later determined that the virion component responsible was the 11kDa protein encoded by the F17 VACV gene (Person-Fernandez & Beaud, 1986). F17 has also been shown to bind to and sequester the mTOR kinase binding proteins Raptor and Rictor, causing mTOR accumulation in the Golgi preventing ISG induction (Meade et al., 2018). It has recently been reported that F17 suppresses the cytosolic DNA sensor cyclic-di-GMP-AMP synthase (cGAS), which mediates IRF responses and ISG activation in VACV lacking F17 (Meade et al., 2019; Sun et al., 2013). Interestingly cGAS or STING knockout did not rescue the defect seen in late-stage viral protein production or inhibit the ISG response seen during infection with VACV lacking F17, suggesting another mTOR regulated process may be involved (Meade et al., 2019). Recently a highly virulent African swine fever virus strain Armenia/07 has been shown to also regulate IFN-β production down through inactivation of the cGAS-STING pathway (García-Belmonte et al., 2019). Inhibiting cytosolic DNA sensors that induce IFN and ISG response would be beneficial for both VACV and ASFV as both DNA viruses replicate predominantly in the cytoplasm (Moss, 2013; Rojo et al., 1999).

VACV protein 169 was recently characterized as a cytoplasmic protein that is expressed as an early gene during viral replication and induces host shutoff by inhibiting translation of both cap-dependent and cap-independent translation of mRNA by an unknown mechanism. Expression of protein 169 in cells caused accumulation of 80S ribosomes and a reduction of polysomes, an indicator of global reduction of protein synthesis. Protein 169 may selectively inhibit host protein synthesis as it was found to be absent from viral factories where viral transcription and translation take place (Katsafanas & Moss, 2007; Strnadova et al., 2015). It was also shown that 169 is capable of inhibiting the synthesis of protein expression linked to immune signaling pathways, but this inhibition was found to be due to general host shutoff, and not selective inhibition. Surprisingly, deletion of 169 from VACV was found to increase virulence as result of increased production of pro-inflammatory cytokines and chemokines, and enhanced recruitment of immune cells to the site of infection (Strnadova et al., 2015).

A group of small, non-translated polyadenylated RNAs termed POLADS have been shown to be associated with VACV induced host shutoff. It was found the poly(A) tail that was responsible for the inhibitory phenotype, and the 5' ends did not contribute to host shutoff and could be from both cellular and viral RNAs (Lu & Bablanian, 1996; Su & Bablanian, 1990). This nonspecific polyadenylation is thought to be a product of the VACV poly(A) polymerase, but evidence of this has not been shown. The size of the POLADS does play a role in the inhibition of protein synthesis, with the larger RNAs having a greater affect when added to a cell free system with HeLa cell mRNAs compared to the smaller POLADS. The inhibition effect of the POLADS had a lesser effect on VACV mRNAs (Su & Bablanian, 1990). The inhibitory effect could be reversed in the cell free system by the addition of purified poly(A) binding protein, and

by purified initiation factor 4A(eIF4A) which is the enzymatic driver of the helicase activity of the eukaryotic initiation factor (eIF) 4F complex (Cacoullos & Bablanian, 1991; Su & Bablanian, 1990).

Coronavirus

Coronavirinae is a large group of enveloped, positive-sense single stranded RNA viruses and have been shown to induce host shutoff upon infection through various mechanisms including disrupting mRNA export, mRNA processing, and mRNA translation (Nakagawa et al., 2016). Coronaviruses are be divided into four genera: alpha, beta, gamma, and delta (Fehr & Perlman, 2015). The most notable of these viruses is SARS-CoV, MERS-CoV, and more recently SARS-CoV-2, which shares 84% sequence identity with SARS-CoV. SARS-CoV-2 infection has shown to interfere with host translation, degrade host mRNAs, and block mRNA export (Finkel et al., 2021)

mRNA Processing and Trafficking. ORF6 contributes to host shutoff by interacting with components of the nuclear pore complex that facilitate RNA export such as Nup98 and Rae1, thus blocking nuclear mRNA export (Addetia et al., 2021; Kato et al., 2021; J. G. Lee et al., 2021). While ORF6 has been shown to inhibit the host antiviral response, the blocking of mRNA export seems to be broad and not selective, as ORF6 reduces the expression of a fluorescent reporter plasmid (Addetia et al., 2021). ORF6 protein has also been to contribute to viral evasion of the Type I interferon antiviral response by sequestering host nuclear import factors and blocking the translocation of stat1/2 into the nucleus and thus preventing induction of IFN-stimulated genes (X. Lei et al., 2020; Miorin et al., 2020). Analysis of COVID19 shows

impaired Type I IFN response during infection and a screening of Sars-CoV-2 viral proteins found Orf6 and Orf8 viral proteins are responsible for the strong inhibition of IFN-β and NF-κB promoter activity, as well as ISRE (Hadjadj et al., 2020; J. Y. Li et al., 2020). This is supported by the observation of upregulation of NF-κB pathway genes from patient samples infected with SARS-CoV-2 carrying a nucleotide deletion in ORF6 (Quéromès et al., 2021). ORF6 shows how a virus can evade the host immune response by utilizing host shutoff mechanisms.

NSP14 protein of SARS-CoV-2 is 99% identical to NSP14 of SARS-CoV that has two enzymatic domains: a 3'-5' exoribonuclease responsible for proofreading RNA transcripts created by the viral RNA-dependent RNA-polymerase and a guanine-N7-methyltransferase that plays a role in the 5' capping of viral mRNA. NSP10 forms a complex with NSP14 and enhances the 3'-5' exoribonuclease activity of NSP14 (Bouvet et al., 2012; Hsu et al., 2021). Though the mechanism has not been elucidated, NSP14 has been shown to induce host shutoff in several human coronaviruses. NSP14 induced host shutoff can be blocked by mutations to either domain and formation of the NSP14-NSP10 complex enhances the host shutoff. Expression of NSP14 did not change the levels or distribution of RNA within the cell, suggesting NSP14 host shutoff mechanism of action may be at translation (Hsu et al., 2021). NSP14 was also shown to block IFN-I dependent ISG induction, and future experiments should elucidate how NSP14 induces host shutoff and helps SARS-CoV-2 evade host antiviral response.

The SARS-CoV-2 NSP16 gene encodes a 2'-O- methyltransferase (2'-O-MTase) that is responsible for modifying viral mRNAs and allowing evasion of host innate immunity. The enzymatic activity of NSP16 is dependent on NSP10, which is known to bind to zinc and RNA

(Bobrovs et al., 2021; Chang & Chen, 2021). Recently the NSP16 protein was shown to bind components of the spliceosome and suppress global mRNA splicing, which contributes to a decrease in mRNA levels upon SARS-CoV-2 infection. This block in splicing was shown to inhibit expression of ISGs using a reporter line with expression of NSP16, and an increase in intron retention in IFN-responsive genes was observed upon SARS-CoV-2 infection (Banerjee et al., 2020).

Translation. The non-structural protein 1 (NSP1) is a multifunctional protein and a major contributor to α- and β-coronavirus induced host shutoff and contributes to evasion of the host innate immunity (Nakagawa & Makino, 2021). Nsp1 of SARS-CoV-2 binds to the mRNA entry channel of the 40S ribosome subunit and globally reduces nascent polypeptide synthesis and accelerates host mRNA degradation (Rao et al., 2021; Schubert et al., 2020; Thoms et al., 2020). In the presence of NSP1, polysome profiling showed a shift from polyribosomes to 80S monosomes, and puromycin pulse-chase analysis showed reduced puromycin incorporation into actively synthesized polypeptides, both hallmark characteristics of host shutoff (Schubert et al., 2020; Thoms et al., 2020). NSP1 has also been shown to directly interact with mRNA export machinery, and when expressed shows an increase in poly(A) RNA levels in the nucleus (K. Zhang et al., 2021). Virus induced host shutoff is thought to not only prioritize translation of viral mRNAs but also inhibit antiviral factors, and NSP1 has been shown to contribute to viral evasion of the host innate immune response as seen with using an IFN-β-promoter firefly reporter assay and measuring endogenous levels if IFN-β and IL-8 when cells are stimulated with Sendai virus (Thoms et al., 2020). NSP1 expression also reduces the phosphorylation of IRF3 and nuclear translocation and reduces the protein levels of Tyk2 and STAT2 antiviral

factors (Kumar et al., 2021). Thus, NSP1 has multiple strategies to induce host shutoff at different levels and down-regulates host innate immunity.

HIV

Human immunodeficiency virus type 1 (HIV-1) is an enveloped positive sense linear RNA retrovirus and is the causative agent of AIDS. HIV is classified in a subgroup of retroviruses, meaning its viral genome is reverse transcribed by viral reverse transcriptase and is transported to the nucleus where it is integrated into the cellular genome as a provirus (Costin, 2007). HIV-1 encodes several mechanisms to evade host immunity and has been shown to induce host shutoff during viral infection (Yin et al., 2020). The HIV-1 virus induced host shutoff was shown along with a decrease in the steady state levels of cellular RNAs, suggesting at least one mechanism works pre-translation (Agy et al., 1990).

mRNA Processing and Trafficking. The HIV-1 virion associated accessory protein Vpr is highly conserved among HIV isolates, simian immunodeficiency virus, and other lentiviruses and is required for efficient viral replication in macrophages and other non-dividing cells (G. Li et al., 2009). Vpr has multiple functions during viral infection involving cytoplasmic nuclear shuttling, induction of cell cycle arrest, regulation of apoptosis, and inhibition of cellular mRNA splicing (Kuramitsu et al., 2005; Miyatake et al., 2016). Vpr associates with the essential splicing factor SAP145 and has been shown to inhibit splicing of cellular mRNA in vivo and vitro (Hashizume et al., 2007). This inhibition of splicing could lead to accelerated degradation of

cellular mRNA and cause a decrease in the steady state levels of cellular RNAs seen during HIV infection.

Translation. The PR protein of HIV-1 is a viral protease that cleaves the retroviral gag-, pol, and Env-encoded proteins that are synthesized as polyprotein precursors (Cimarelli & Luban, 1999). Viral proteases can also target host proteins to disrupt cellular functions, and the PR protein has been shown to cleave various cellular proteins contributing to viral evasion of host innate immunity (Rajput et al., 2011; R. N. Wagner et al., 2015). Recently, it was shown that PR can cleave TBK1 and disrupt downstream signaling and IFN induction (Jeremiah et al., 2021). In addition to targeting proteins involved in innate immunity signaling pathways, the HIV-1 PR protein has also been shown to induce host shutoff by cleavage of the translation initiation factor eIF4G and PABP (Alvarez et al., 2006; Ventoso et al., 2001). This cleavage strongly inhibits cap-dependent translation of cellular mRNA and stimulates the cap-independent translation of HIV-1 viral mRNAs that utilizes an internal ribosome entry site (Castelló et al., 2009; Locker et al., 2011). Multiple viruses have been found to encode internal ribosome entry sites to attract initiation complexes independent of the 5'-cap.

The HIV-1 Gag polyprotein contains four major structural domains that are processed into their mature form by the HIV-1 viral protease PR. The mature products include the MA(matrix), CA (capsid), NC (nucleocapsid), and p6 domains (Cimarelli & Luban, 1999). These domains interact with numerous host proteins and have a critical role early on in the viral life cycle and during virion assembly. A comprehensive review on the HIV-1 Gag interactions and manipulation of host proteins was recently provided by Klingler et al. (2020). The MA

domain was shown to interact with the translation elongation factor EF1α through a yest two-hybrid screening, and this interaction was shown to disrupt translation, including translation of viral mRNA, which is thought to release viral mRNA to be packaged into virions (Cimarelli & Luban, 1999)

Summary and Future Directions

A virus can encode multiple factors that interfere at different stages of gene expression from transcription, mRNA maturation and export, to translation. Each stage of gene expression can be considered an essential process and highly interconnected with one another. The flow of genetic information is also vital for the host cell to respond to various stresses such as viral infection and disruption of gene expression can result in a reduced antiviral response. This makes host shutoff factors desirable targets for the development of new therapeutics or for creating attenuated viruses. Because viruses may employ multiple mechanisms to induce host shutoff, more work needs to be done and new techniques developed to identify novel host shutoff factors and characterize their role in the suppression of host translation.

The work presented in this dissertation demonstrates the efficacy of a novel high-through put *Gaussia* luciferase assay as a tool to study host translation suppression by viral infection, as well as identifies new viral host shutoff factors of VACV and SARS-CoV-2 expanding the current understanding of virus-host interactions. Future research will continue to characterize previously identified host shutoff factors and identify new host shutoff factors as well as new mechanisms and pathways that viruses manipulate to suppress host cellular translation.

Chapter 2 - Rapid and Quantitative Evaluation of Vaccinia Virus-Induced Host Shutoff Using Newly Generated Cell Lines Stably Expressing Secreted *Gaussia* Luciferase

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Abstract

Host shutoff, characterized by a global decline of cellular protein synthesis, is commonly observed in many viral infections, including vaccinia virus (VACV). Classic methods measuring host shutoff include the use of radioactive or nonradioactive probes to label newly synthesized proteins followed by radioautography or sodium dodecyl-sulfate polyacrylamide gel electrophoresis to resolve the proteins for follow-up detection. Although these are highly reliable methods, they are time- and labor-consuming. Here, we generated two cell lines stably expressing secreted *Gaussia* luciferase. These reporter cells allow rapid, quantitative, and consecutive monitoring of host shutoff from a single infection sample. We evaluated host shutoff induced by wild-type and various mutant VACVs using the reporter cell lines. The results validated the utilities of the reporter cells and quantitatively

characterized VACV-induced host shutoff at different stages of replication. Notably, the results also indicated additional major unidentified VACV shutoff factors. Our study provides new tools to study host shutoff. The reporter cells are also suitable for high throughput settings and rapid testing of clinically isolated viruses. In combination with classical methods, these tools will greatly facilitate understanding of virus-induced host shutoff, and protein synthesis shutoff caused by other physiologically relevant stresses.

Keywords: *Gaussia* luciferase, host shutoff, poxvirus, reporter cell line, vaccinia virus

Pervasive downregulation of cellular gene expression featured by a global shutdown of nascent protein production is commonly observed when cells experience various stresses, including many viral infections. This virus-induced reduction in global protein synthesis is often termed "host shutoff." Virus-encoded factors utilize various mechanisms that can interfere at different stages of the gene expression such as transcription, mRNA processing, mRNA stability, and translation. Virus-induced host shutoff redirects the cellular metabolites to build viral macromolecules and can also disrupt host antiviral responses as these responses often require the synthesis of new effector proteins (Dhungel et al., 2020).

Vaccinia virus (VACV) is the prototypic member of poxviruses that comprise a large family of double-stranded DNA viruses (Moss, 2013). Despite the eradication of smallpox, the most notorious poxvirus disease caused by the variola virus (Thèves et al., 2016), there are still many other poxviruses currently endemic and cause significant diseases in humans and other animals (Erez et al., 2019; Ng et al., 2019; Reed et al., 2004; Vaughan et al., 2018). Moreover,

poxviruses are actively developed as vaccine vectors and for oncolytic virotherapy (Altenburg et al., 2014; Breitbach et al., 2011; Garcia-Arriaza & Esteban, 2014; Izzi et al., 2014; Pugalenthi et al., 2015; Rerks-Ngarm et al., 2009; Wiktor et al., 1984; Wiktor et al., 1992; Yang et al, 2021). VACV has been known to cause profound protein synthesis shutoff in infected cells and is a model for studying virus-induced host shutoff (Becker & Joklik, 1964; Moss, 1968; Moss & Salzman, 1968). Multiple ways by which VACV induces host shutoff have been identified, including transcriptional inhibition, mRNA export, mRNA stability, and translation, as well as posttranslational protein degradation (Dhungel et al., 2020). Particularly, two decapping enzymes, D9 and D10, that remove mRNA 5′cap and induce mRNA degradation, are believed to be significant contributors to VACV-induced host shutoff (Cantu et al., 2020; Cao et al., 2022; Parrish & Moss, 2006, 2007; Parrish et al., 2007). These studies do not exclude other major host shutoff factors encoded by VACV remaining to be identified.

Pulse labeling of nascent proteins using radioactive amino acids is the gold standard method to measure host shutoff (Pollard, 1994). In this method, cells are first starved of amino acids, then the addition of radioactively labeled amino acids in the culture medium for a period of time, followed by autoradiography to quantify the radioactivity or Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to resolve the proteins and then visualize by phosphoimager. However, this method involves biohazardous radioactive materials, which require a permit and special equipment to be used in a contained environment. Moreover, it has been shown this type of radiolabeling can cause DNA damage, induce cell cycle arrest, alter cell morphology, and induce apoptosis (Hu & Heikka, 2000; Hu et al., 2001), which is unfavorable for some experiments. Methods without the involvement of radioactive isotopes have been

developed and are more frequently used. For example, click chemistry-based pulse labeling allows the attachment of fluorophore or other reporter molecules to newly synthesized proteins (Best, 2009). Puromycin, an aminonucleoside antibiotic that can be incorporated at the C-termini of newly synthesized proteins, is another often used molecule to label nascent proteins to be detected by anti-puromycin antibodies (Goodman & Hornberger, 2013; Schmidt et al., 2009). Although these radioactive and nonradioactive methods have extensively advanced the studies of virus-induced host shutoff, they are time- and labor-consuming. They are not well suited for studying a large number of subjects simultaneously due to relatively complex experimental settings. Furthermore, they are unsuitable for rapid testing of clinically isolated viruses. New methods allowing rapid and quantitative monitoring of host shutoff are needed.

Here, we developed two cell lines stably expressing naturally secreted bioluminescent *Gaussia* luciferase (GLuc), allowing monitoring host shutoff rapidly, quantitatively, and consecutively from the media of a single infection sample. Using these reporter cell lines, we systemically evaluated VACV-induced host shutoff. The results validated the utilities of the reporter cells in studying host shutoff and quantitatively measured the contributions of VACV-induced host shutoff by the events in different replication stages. The results also suggested additional unidentified major VACV shutoff factors. Overall, our study provided valuable tools in host shutoff research and revealed important features of VACV-induced host shutoff.

Materials and Methods

Cells and Viruses

A549, A549DKO (with PKR and RNase L knocked out), A549-EF1α- Gluc, and A549DKO-EF1α-Gluc cells were cultured in Dulbecco's minimal essential medium (DMEM; Quality Biological) supplemented with 10% fetal bovine serum (FBS; Peak Serum), 2 mM glutamine (Quality Biological), 100 U/ml of penicillin (Quality Biological), and 100 μg/ml streptomycin (Quality Biological). The cells were grown under 5% CO_2 at 37°C. A549 and A549DKO were kind gifts from Dr. Bernard Moss (R. Liu & Moss, 2016). A549-EF1α-Gluc and A549DKO-EF1α-Gluc were generated in this study.

All VACVs used in this study are based on VACV Western Reserve (WR) strain (ATCC VR-1354). vD9muD10mu, vD10mu, vΔD10, vΔD9, vD9mu, vΔA23, and vRO-G8R were kind gifts from Dr. Moss (S-W. Liu et al., 2015; S-W. Liu et al., 2014; Warren et al., 2012; Y. Zhang et al., 1992). Wild-type (WT) VACV, vD10mu, vΔD10, vΔD9, vD9mu, and vD9muD10mu were prepared and titrated as described elsewhere (Cantu et al., 2020; Cotter et al., 1998; S-W. Liu et al., 2015).

VACV infection was carried out using DMEM containing 2.5% FBS. The virus was sonicated and diluted according to desired MOI. The medium containing viruses was added to the cultured cells and incubated at 37°C for 1h and replaced with fresh medium.

Chemicals and Antibodies

Puromycin (10191-150), anti-puromycin antibody (MABE343), Isopropyl beta-D-thiogalactopyranoside (IPTG, I6758), and Cytosine- 1-β-D-arabinofuranoside (AraC, C1768) were purchased from Sigma-Aldrich. Lipofectamine 2000 Reagent (11668-019) was purchased from Invitrogen. Opti-MEM Reduced Serum Medium (31985-070) used in transfection was purchased from Thermo Fisher Scientific.

Generation of Plasmid and Cells Lines With Stable Expression of *Gaussia* Luciferase

The pEF1α-Gluc reporter plasmid used to generate the stably expressing cell lines were generated by inserting the constitutive cellular promoter from the human elongation factor 1 alpha (EF1α) into a *Gaussia* Luciferase vector containing a promoter-multiple cloning site (pMCS) upstream of the naturally secreted bio-luminescent *Gaussia* luciferase gene (from Thermo Fisher Scientific) using an In-Fusion HD cloning kit from Takara Bio (639649) per manufacturers protocol. Correct insertion of EF1α was verified by sequencing.

To generate the stable cell lines, the resulting pEF1α-Gluc reporter plasmid was transfected using Lipofectamine 2000 Reagent into A549 human lung carcinoma cells or A549DKO cells per the manufacturer's protocol. Twenty-four hours posttransfection, the transfected cells were amplified, and 48h posttransfection or when the cells were confluent, the media was replaced with selective media containing 0.6 μg/ml of puromycin. Cells were gently washed with phosphate-buffered saline to remove any dead cells and selective media was replaced every 24h. After 10 days to 2 weeks, when clusters of cells containing the antibiotic resistance became visible, cells from single colonies were collected and amplified and the

supernatant was collected for *Gaussia* Luciferase assay analysis | Nascent protein synthesis analysis and Western blot analysis.

The puromycin labeling method to detect nascent protein synthesis was described previously (Pant et al., 2019). About 10 μg/ml of puromycin is added to the cells for 20 min followed by harvesting for Western blot analysis using anti-puromycin antibodies. Western blot was carried out as described previously (Pant et al., 2021). Samples were resolved using SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF). The PVDF membrane was then blocked with 2% bovine serum albumin for 1h, incubated with primary antibodies for 45 min, washed with TBST three times, incubated with secondary antibodies for 1h, and washed three times with TBST. All the steps were carried out at room temperature. The membrane was developed using a chemiluminescent substrate and visualized using an Azure c300 bioimaging system.

Gaussia Luciferase Activity Assay

Gaussia luciferase activities were measured using the Pierce *Gaussia* luciferase flash assay kit (Thermo Fisher Scientific, 16158) by a luminometer, according to the manufacturer's instructions.

Statistical Analysis

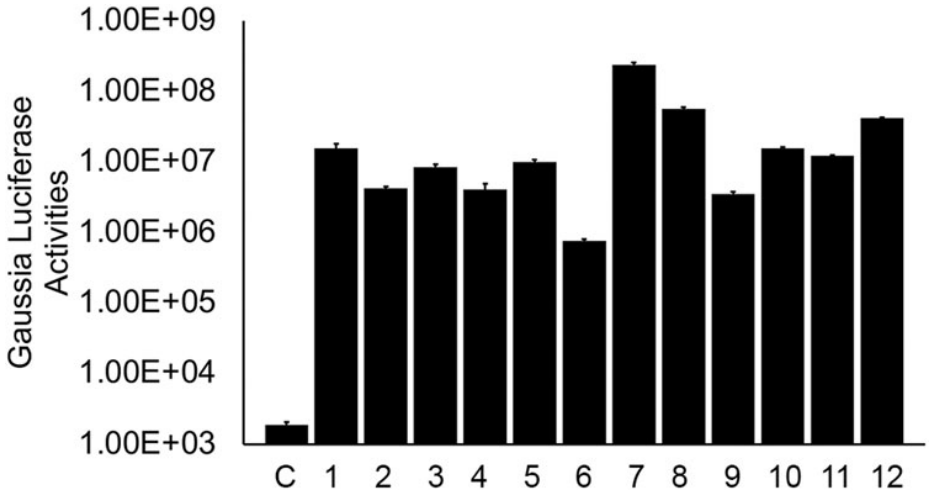
Student's *t*-test was performed to evaluate statistical differences from at least three replicates. The following symbols were used to indicate statistical significance: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; **** $p \leq 0.0001$.

Results

Generate an A549 Cell Line Stably Expressing *Gaussia Luciferase*

Gaussia luciferase naturally secretes into the extracellular environment (Maguire et al., 2009; Verhaegent & Christopoulos, 2002), which can be measured by taking a small amount of the cell culture medium at given times, allowing monitoring gene expression rapidly, quantitatively, and consecutively from a single sample. We first constructed a reporter plasmid by inserting the constitutive human cellular elongation factor 1 alpha (EF1α) promoter upstream of the *Gaussia* luciferase gene of a plasmid vector. We then generated A549-EF1α-Gluc stably expressing the *Gaussia* luciferase gene by transfecting the EF1α-Gluc reporter plasmid into A549 cells, a human lung carcinoma cell line, and selecting clones under antibiotic selection pressure. Lung cells are physiologically relevant as VACV replicates at high titers in the lungs of infected animals (Sood et al., 2008). In fact, many other viruses also infect lung cells. We isolated 12 colonies of A549-EF1α-Gluc and tested their *Gaussia* luciferase activities (see Figure 2.1). While there were variations, in general, high *Gaussia* luciferase activities were observed from the media of these cell colonies. No discernable differences in cell growth or morphogenesis were noticed among these colonies.

Figure 2.1



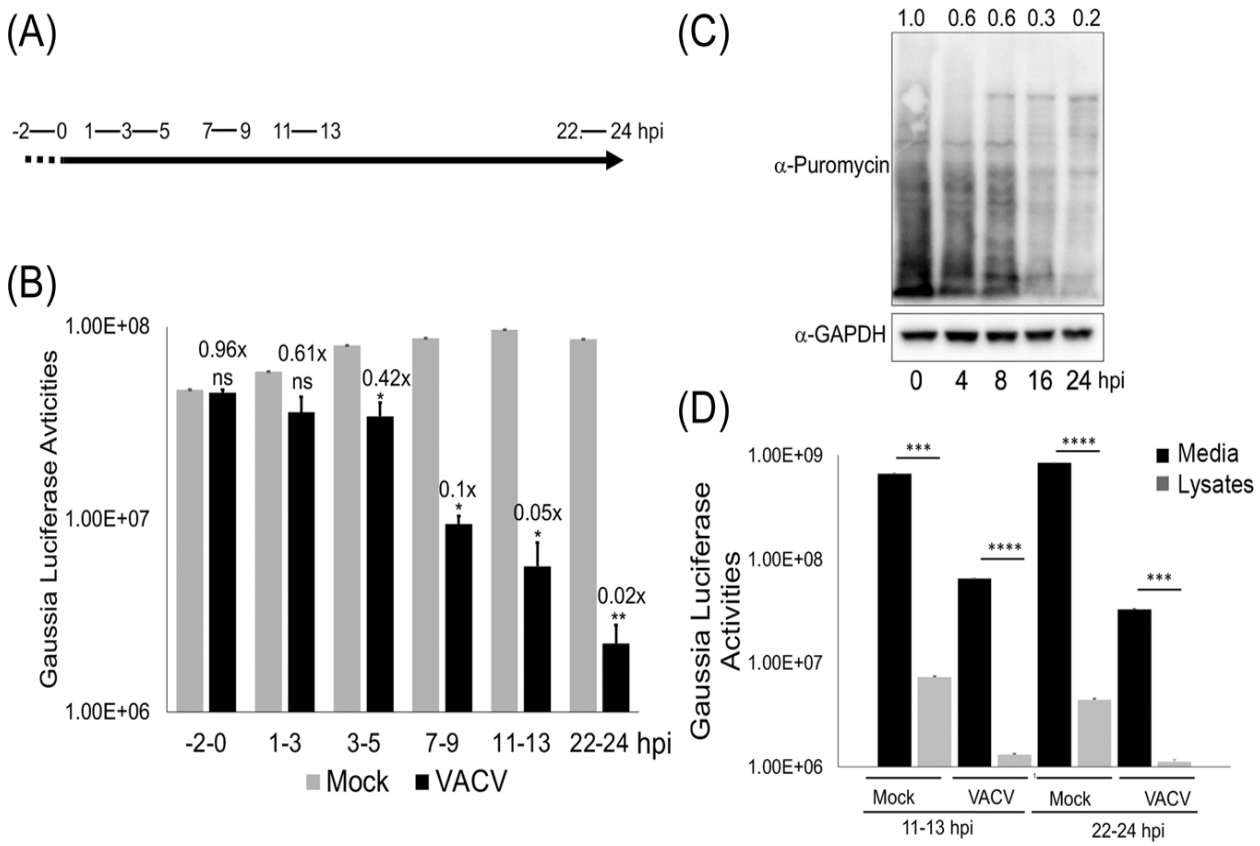
Generation of A549-EF1α-Gluc Cell Line Stably Expressing Gaussia Luciferase. Twelve colonies of A549-EF1α-Gluc cells were isolated and amplified. The *Gaussia* luciferase activities from the cell culture media were measured using a luminometer. Error bars indicate standard deviations of three replicates. C: control media.

Monitor Host Shutoff Rapidly, Quantitatively, and Consecutively From a Single Infected Well Using A549-EF1α-Gluc

We rationalized that the gene expression during a specific time period during VACV infection could be measured by replacing the culture media with fresh media and incubating for a desired period of time. The *Gaussia* luciferase activities from the newly added media reflect nascent *Gaussia* luciferase protein during the specific time window, allowing the assessment of VACV-induced host shutoff consecutively. We next evaluated VACV-induced host shutoff using the #7 A549-EF1α-Gluc cell line clone with strong *Gaussia* luciferase activity at different time points during infection. We infected A549- EF1α-Gluc with VACV, and the cell media

was collected and replaced after each time window: −2 to 0, 1–3, 3–5, 7–9, 11–13, and 22–24 hpi (see Figure 2.2). The results showed a significant reduction of *Gaussia* luciferase activities starting from the 3 to 5 hpi time window, with an over twofold reduction. The reduction folds increased over time. During the 22–24 hpi time window, a ~50-fold reduction was observed (Figure 2.2-B). These results were consistent with what had been previously seen in the literature, with a greater reduction of host protein synthesis at the later stages of VACV infection (Beaud & Dru, 1980). We also tested the stable cell lines #1, 8, 10, and 12 for the time window 23–24 hpi and found a significant reduction in *Gaussia* luciferase activities in these cell lines (data not shown).

Figure 2.2



Monitor VACV-Induced Host Shutoff Rapidly, Quantitatively, Consecutively, and in Real-Time From One Single Sample Using the A549 Gaussia Luciferase Reporter Cell Line A549-EF1α-Gluc. (A) Schematic representation of the time course measuring *Gaussia* luciferase activities during VACV infection of A549-EF1α-Gluc cells. Two hours before each collection, the cells were replenished with fresh media and incubated for 2h, followed by *Gaussia* luciferase assay. (B) *Gaussia* luciferase activities in media of the cultured A549-EF1α-Gluc cells infected with VACV or mock-infected. *Gaussia* luciferase activities from several 2h windows over the course of infection were shown. Numbers indicate the reduction of *Gaussia* luciferase compared to the mock in each group. Significance was compared to the mock. (C) A549 cells were infected with VACV at an MOI of 5. Cells were treated with 10 μg/ml puromycin for 20 minutes before collection at indicated time points, followed by Western blot analysis using α-puromycin and α-GAPDH antibodies. The numbers above the lane indicate GAPDH-normalized intensities. The image is a representative of three replicates. (D) *Gaussia* luciferase activities in media and cell lysates of the cultured A549-EF1α-Gluc cells infected with VACV or mock-infected. *Gaussia* luciferase activities (normalized with the total volume of samples from media and lysates) from the indicated 2h windows during infection were shown. Error bars indicate the standard deviations of at least three replicates: VACV, vaccinia virus. ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

An alternative to studying nascent protein synthesis by radioactive isotope labeling utilizes puromycin incorporation into newly synthesized, prematurely terminated proteins to monitor protein synthesis. We carried out the puromycin labeling analysis by measuring nascent protein synthesis during VACV infection and comparing it to the *Gaussia* luciferase reporter assay. The results showed a decrease in nascent protein intensities in infected cells over the course of VACV infection, with the later time points showing an increased reduction compared to the earlier time points, a trend similar to what we observed in *Gaussia* luciferase activities using the A549-EF1α-Gluc reporter cell line (Figure 2.2-C). The decrease of the puromycin signal intensities over the course of VACV infection was less prominent than in A549-EF1α-Gluc, indicating that the *Gaussia* luciferase activity measurement is more sensitive. Together, these results validated the A549-EF1α-Gluc as an effective tool to analyze VACV-induced host shutoff rapidly and quantitatively.

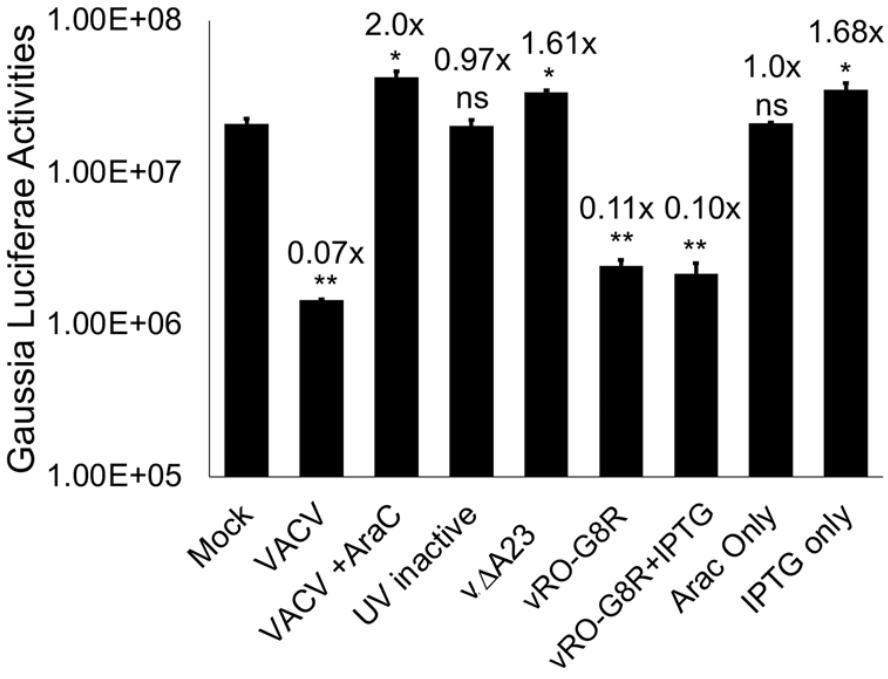
We have previously shown that *Gaussia* luciferase secretion is not affected by VACV infection of HeLa cells using a transfected plasmid (Peng et al., 2020). We measured the *Gaussia* luciferase activities in media and cell lysates, respectively, to rule out the possibility that *Gaussia* luciferase was retained in the A549-EF1α-Gluc cells due to VACV infection. The results indicated that (i) the vast majority (~99%) of *Gaussia* luciferase activities were in the media, and (ii) in both the media and the cell lysates, VACV infection led to significantly reduced *Gaussia* luciferase activities (Figure 2.2-D). Overall, we concluded the A549-EF1α-Gluc cell line was a valuable new tool to quantify host shutoff in virus-induced cells in real time.

Post-DNA Replication Events Dominate VACV-Induced Host Shutoff

VACV gene expression has three stages based on their expression timing: early, intermediate, and late. The early genes initiate their expression immediately after VACV enters the cells before viral DNA replication. In contrast, intermediate and late genes need to use replicated viral DNA as templates to initiate their transcription (Moss, 2013). Multiple factors were shown to cause VACV-induced host shutoff, including proteins incorporated in virions and proteins expressed at the early and post-replicative (intermediate and late) stages (Dhungel et al., 2020). We used A549-EF1α-Gluc cells to determine the contributions of different stages of VACV replication to the host shutoff using various VACV mutant viruses and chemicals that block VACV infection at specific stages of replication. UV-inactivated VACV (blocking VACV early gene expression due to damaged viral DNA), cytosine arabinoside (AraC) treatment (blocking VACV DNA replication; Renis & Johnson, 1962), and v*Δ*A23 (with deletion of

intermediate transcription factor A23, blocking VACV intermediate gene expression but allowing viral DNA replication; Warren et al., 2012) did not cause decreased *Gaussia* luciferase activities (Figure 2.3). In contrast, vRO-G8R (with IPTG-inducible viral late transcription factor G8) caused a significant decrease in *Gaussia* luciferase activities no matter whether IPTG was added. In vRO-G8R, VACV late genes are expressed under the inducible control of IPTG, and in the absence of IPTG only early and intermediate genes are expressed (Y. Zhang et al., 1992). These results indicated that the intermediate stage events were sufficient for inducing a profound host shutoff, while it does not exclude the contribution of the late- stage events.

Figure 2.3



Post-DNA Replication Events Dominate VACV-Induced Host Shutoff. Gaussia luciferase activities between 13 and 15 hpi of the cultured A549-EF1α-Gluc cells infected with indicated viruses or treatments. Numbers indicate the reduction/increase of *Gaussia* luciferase activities compared to mock. Error bars indicate the standard deviations of at least three replicates: VACV,

vaccinia virus. ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$. Significance was compared to the mock.

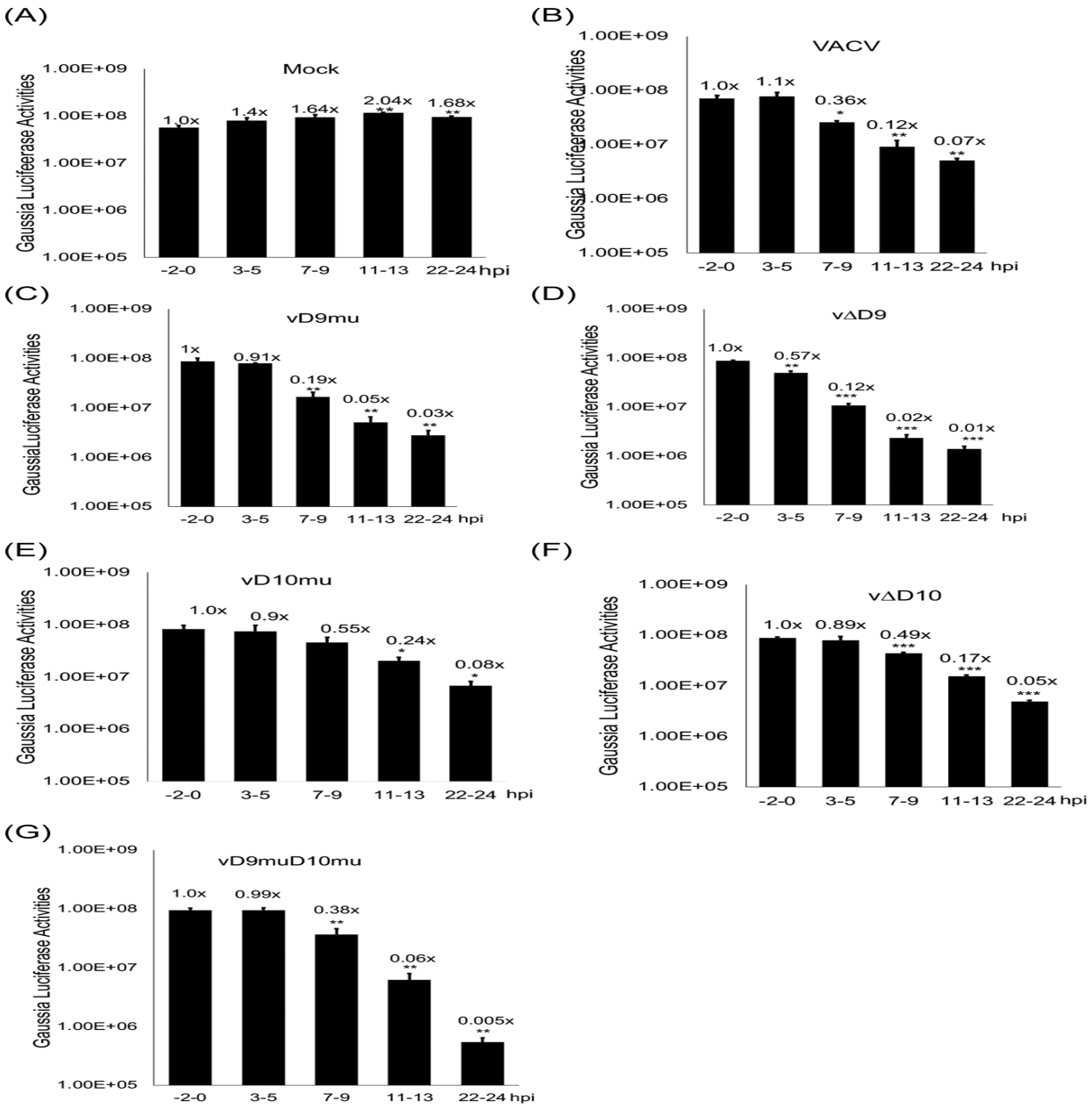
VACV With Inactive/Deleted Decapping Enzymes Retains the Ability to Induce Profound Host Shutoff

VACV-encoded decapping enzymes (D9 and D10) that cause accelerated mRNA degradation are the most well-studied VACV host shutoff factors (Parrish & Moss, 2006, 2007; Parrish et al., 2007; S-W. Liu et al., 2015; S-W. Liu et al., 2014). D9 has been found to be non-essential for viral replication in cultured cells, but deletion of D10 results in smaller plaque size and a reduction in replication (Cao et al., 2022). This correlates with our observation of early gene expression having little effect on inducing host shutoff, (Figure 2.3) as D9 is an early viral gene and D10 is an intermediate gene expressed post-DNA replication where we see greater host shutoff (Parrish & Moss, 2007; Yang et al., 2010, 2011). This would suggest that the post-replicative gene D10 plays a more critical role than D9 to induce host shutoff. We next evaluated VACV-induced host shutoff using recombinant viruses with inactive/deleted one or both of the decapping enzymes. vD10mu and v⊿D10 are recombinant VACVs with the D10 Nudix motif (the decapping activity-harboring motif) mutated and D10 knocked out, respectively. v⊿D9 and vD9mu are recombinant VACVs with the D9 Nudix motif mutated and D9 knocked out, respectively. vD9muD10mu contains inactive D9 and D10 with the Nudix motif mutated that do not decap RNA (S-W. Liu et al., 2015; S-W. Liu et al., 2014). *Gaussia* luciferase activities were measured during −2 to 0, 3–5, 7–9, 11–13, and 22–24 hpi time windows. Although the reduction levels varied, significant reductions of *Gaussia* luciferase activities were observed in all the infections with deleted or inactivated decapping enzymes at the later stages of infections (see Figure 2.4). Interestingly, at the relatively early time windows,

3–5 and 7–9 hpi, we observed the highest host shutoff in vΔD9 and vD9mu infections when compared to v⊿D10 and vD10mu infection, suggesting an earlier effect of D9 that is expressed early VACV infection.

Although viral factors contribute to host shutoff, cellular responses to viral infections may also contribute to this phenomenon. In the absence of active decapping enzymes during VACV infection, double-stranded RNAs (dsRNAs) are accumulated in infected cells, leading to the activation of RNase L and PKR pathways that are known to induce RNA decay and inhibit protein synthesis, respectively (R. Liu & Moss, 2016). The strong host shutoff induced by VACV in A549-EF1α-Gluc with inactivated/deleted decapping enzymes is at least partially due to these mechanisms. To examine the PKR- and RNase L-independent mechanisms induced by VACV, we generated A549DKO-EF1α-Gluc using the A549DKO as the parental cells. The PKR and RNase L gene loci were knocked out from the A549 by CRISPR/Cas9 (R. Liu & Moss, 2016), providing a valuable tool to study VACV infection as the activated PKR and RNase L trigger RNA degradation and translation repression were excluded, allowing us to determine if there are additional host shutoff factors whose effects may be masked when PKR and RNase L are present. Similar experiments to Figure 2.4 were carried out using an A549DKO-EF1α-Gluc stably expressing cell line that had comparable *Gaussia* luciferase activities to that of A549-EF1α-Gluc in the mock-infection condition (see Figures 2.4-A and 2.5-A).

Figure 2.4



VACV With Inactive/Deleted Decapping Enzymes Still Induces Host Shutoff Profoundly in A549-EF1a-Gluc. Gaussia luciferase activities from media of cultured A549-EF1α-Gluc cells with mock-infected (A), infected with WT-VACV (B), D9mu (C), ΔD9 (D), D10mu (E), ΔD10 (F), and vD9muD10mu (G) at the indicated time windows over the course of infection. Numbers indicate the reduction of *Gaussia* luciferase compared to the time (−2 to 0) hpi window. Error bars indicate the standard deviations of at least three replicates: VACV, vaccinia virus. ns, $p >$

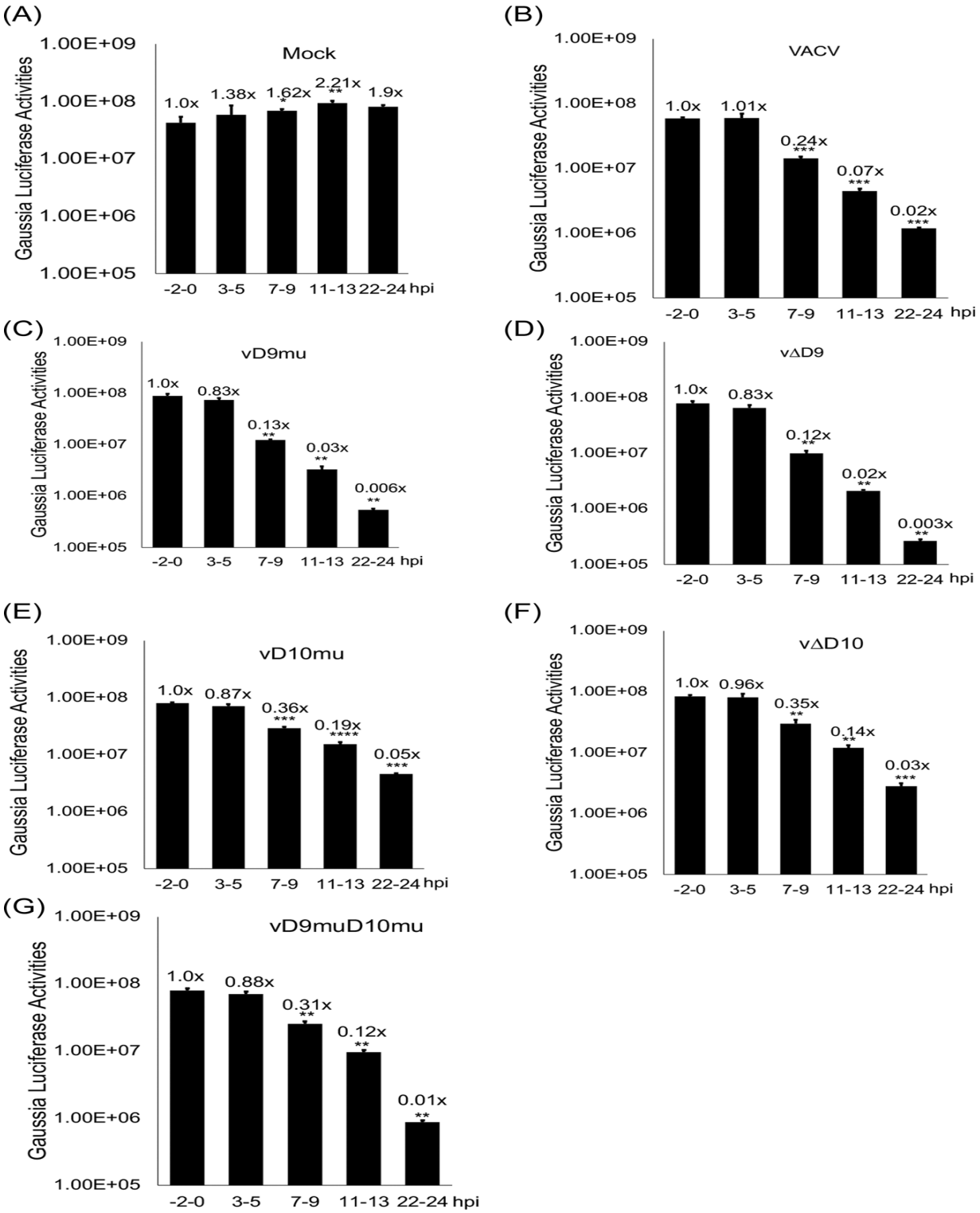
0.05; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; $0.0001 < p \leq 0.001$. Significance was compared to the (−2 to 0) hpi time window.

Again, significant reductions of *Gaussia* luciferase activities were observed in all the VACV infections at the late stages of infections (see Figure 2.5-A–G), indicating decapping-, PKR-, and RNase L-independent mechanism(s) exist to induce host shutoff during VACV infection. Interestingly, we also observed higher host shutoff during the 7–9 hpi time window for A549DKO-EF1α- Gluc with vΔD9 or vD9mu infection (see Figure 2.5-C–F).

Discussion

In this study, we developed two cell lines stably expressing naturally secreted *Gaussia* luciferase based on the human lung carcinoma cell A549. These reporter cell lines have several advantages over classical methods that utilize either radioactive or nonradioactive probes to label nascent proteins in studying host shutoff. First, the detection of the secreted *Gaussia* luciferase is rapid as it does not involve lengthy SDS-PAGE, radioactivity exposure, or Western blot analysis. The secreted *Gaussia* luciferase can be measured directly by taking a very small amount of culture medium and measuring bioluminescence using a luminometer. Second, while the use of radioactive or nonradioactive probes could be quantitated or semi-quantitated based on the signal intensities, *Gaussia* luciferase provides a more sensitive quantitation in a digitized manner. Third, the *Gaussia* luciferase activities from different time points over the course of the experiments can be measured from the same sample consecutively, while the classical methods have to use different samples for different time points.

Figure 2.5



VACV With Inactive/Deleted Decapping Enzymes Still Profoundly Induces Host Shutoff in A549DKO-EF1a-Gluc Cells With Deficient PKR and RNase L Pathways. Gaussia luciferase

activities from media of cultured A549DKO-EF1α-Gluc cells with mock-infected (A), infected with WT-VACV (B), D9mu (C), ΔD9 (D), D10mu (E), ΔD10 (F), and vD9muD10mu (G) at the indicated time windows over the course of infection. Numbers indicate the reduction of *Gaussia* luciferase compared to the time (−2 to 0) hpi window. Error bars indicate the standard deviations of at least three replicates: VACV, vaccinia virus. ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$. Significance was compared to the (−2 to 0) hpi time window.

Compared to plasmid transfection-based reporter systems, the cell lines with the luciferase gene integrated into the genome better mimics what happens to endogenous cellular genes. The biggest caveat of these reporter cell lines is that only one promoter and gene are being measured, which may not reflect the global picture of host shutoff under some circumstances. Therefore, these cell lines are suitable for rapid initial investigation. They are also suitable for high throughput screening, followed by verification by classical or alternative methods. These cell lines are also suitable for studying the well-established host shutoff's molecular mechanisms. The generation of the A549DKO-EF1α-Gluc stably expressing cell line allows us to discover new mechanisms, both viral and cellular, that contribute to host shutoff independent of PKR and RNase L pathways, and this concept may be applied to other host shutoff factors to further advance the field of study. Thus, the reporter cell lines add valuable new tools to monitor host shutoff rapidly, quantitatively, consecutively, and in real-time.

Using these cell lines, we quantitatively evaluated VACV-induced host shutoff. Many viral factors had been suggested to contribute to VACV-induced host shutoff, including factors incorporated in the virions (Hanafusa, 1960; Person et al., 1980), proteins expressed at the early replication stage (e.g., D9 and VACWR169; S-W. Liu et al., 2015; Parris & Moss, 2007; Strnadova et al., 2015), and proteins expressed after viral DNA replication (e.g., D10 and F17; Cantu et al., 2020; Parish & Moss, 2006; Parish et al., 2007; S-W. Liu et al., 2014; Meade et al.,

2019; Person et al., 1980). They have been found to inhibit cellular transcription, interfere with mRNA processing, speed up mRNA degradation, or suppress mRNA translation. We comprehensively reviewed this topic recently (Dhungel et al., 2020). Interestingly, our study using recombinant VACVs or chemical inhibitors to arrest VACV replication at various stages of replication indicate that the events at or before viral DNA replication, including virion-associated factors, contribute minimally to VACV-induced host shutoff, while the post-replicative stage, including intermediate and late replication stages, dominates the host shutoff induction (see Figure 2.3). It is also interesting that the intermediate stage is sufficient to induce the shutoff, although it does not exclude the late events. VACV-encoded decapping enzymes, D9 and D10, which cause mRNA degradation, are significant contributors to VACV-induced host shutoff (Parrish & Moss, 2006, 2007; Parrish et al., 2007; S-W. Liu et al., 2015; S-W Liu et al., 2014). As D9 is expressed at the early viral replication stage and D10 is expressed at the intermediate stage (Yang et al., 2010; Yang et al., 2011), it suggests that D10 plays a more important role in VACV-induced host shutoff. Notably, the findings using various D9 and D10 deletion/mutation mutants indicate that VACV encodes additional major host shutoff factors as these decapping enzymes inactivated VACVs still cause profound *Gaussia* luciferase activity downregulation in A549-EF1α-Gluc and A549DKO-EF1α-Gluc cells (see Figures 2.4 and 2.5). Future investigations will identify additional host shutoff factors. The reporter cell lines developed here in this study will be very useful tools to screen VACV post-replicative genes for their roles in VACV-induced host shutoff.

In summary, we developed two secreted *Gaussia* luciferase-based cell lines that allow us to monitor VACV-induced host shutoff rapidly, quantitatively, consecutively, and in real time.

We used these cells lines and verified known and previously undiscovered new features of VACV-induced host shutoff. The cell lines provide new tools to understand host shutoff and identify additional VACV host shutoff factors, as well as shutoff caused by other virus infections and other stresses.

Author Contributions

Zhilong Yang: Conceptualization; formal analysis; funding acquisition; investigation; project administration; supervision; visualization, writing – original draft; writing – review and editing. Joshua A. Molina: Conceptualization; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author.

Chapter 3 - Identification of Novel Vaccinia Virus Host Shutoff Factors

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Abstract

Host shutoff is the phenomenon observed when many viruses downregulate host cellular gene expression resulting in a global suppression of nascent protein production. A virus can encode multiple factors that interfere at different stages of gene expression from transcription, mRNA maturation and export, to translation. Previous work of our lab indicates additional previously unidentified factors of vaccinia virus host shutoff that are expressed after viral DNA replication. In this study we developed a VACV late gene screening assay to identify additional viral late genes that contributed to host shutoff. We created a reporter plasmid that has the constitutive human cellular elongation factor 1 alpha (EF1α) promoter inserted upstream of a *Gaussia* luciferase gene of a plasmid vector (EF1α-*Gaussia*). Utilizing EF1α-*Gaussia* reporter plasmid we identified eight VACVWR late genes that were capable of inducing host shutoff. We also showed that these genes were capable of reducing *Gaussia* mRNA levels. Next, we designed codon-optimized plasmids of four VACVWR late genes that showed strong translation inhibition and showed that the genes were capable of inducing host shutoff outside the context of infection. The results of this study indicate additional previously unknown VACV host shutoff factors. This work contributes to our understanding of viral host shutoff, and future investigations will elucidate the mechanisms and selectivity of the suppression of cellular translation.

Keywords: Vaccinia virus, poxvirus, *Gaussia* luciferase, Host shutoff

Introduction

As viruses depend on host cell translation machinery to synthesize viral proteins required for viral replication, many have developed various mechanisms to interfere with the expression of cellular genes resulting in a global depression of host protein synthesis termed host shutoff. Host shutoff not only has been shown to redirect cellular metabolites for viral reproduction but has also been shown to evade the host cell antiviral responses (Dhungel et al., 2020).

Vaccinia virus (VACV) is a member of *Poxviridae*, a family of large double-stranded DNA viruses that replicate entirely in the cytoplasm that can divided into two subfamilies based on vertebrate and insect host range, termed Chordopoxvirinae and Entomopoxvirinae, respectively (Moss, 2013). Poxviruses that infect both animals and humans such as ORF virus, cowpox and monkeypox can cause human disease, while other poxviruses such as Capripoxvirus can infect cattle, sheep, and goats and can result in devastating economic losses (Yang et al., 2021). VACV is known to cause host shutoff through various strategies including disruption of transcription, mRNA processing, and translation initiation, and is a model for studying virus-induced host shutoff (Dhungel et al., 2020). The most well studied VACV host shutoff factor is the decapping enzyme D10 (VACWR115), which is known to accelerate the degradation of mRNAs through the removal of their 5' cap. The rapid turnover of mRNA by D10 has been shown to play a role in immune evasion as it prevents accumulation of dsRNA blocking host anti-viral responses (Burgess & Mohr, 2015; S-W. Liu et al., 2015).

While D10 is thought to be a major contributor to VACV-induced host shutoff, other viral genes have been shown to induce host shutoff opening up the possibility that some viral genes that contribute to host shutoff have yet to be identified. Recent work in our lab (Molina & Yang, 2022) has shown that post-replicative processes are mainly responsible for host shutoff, so we designed an assay utilizing naturally secreted *Gaussia* luciferase reporter plasmid based on unique poxvirus biology to screen the post-replicative genes of VACV for their ability to induce host shutoff. This preliminary screening identified 8 VACV post-replicative genes that were capable of reducing *Gaussia* luciferase activity by >70%. A common theme between these genes were their association with the membrane involved in the secretory pathway. Many host shutoff factors are involved in the induction of host shutoff either pre-translation or translation directly, this study suggests that interference of post-translational processes such as protein trafficking may contribute to host shutoff. We further characterized the reduction in *Gaussia* luciferase activities by the post-replicative genes in an attempt to further elucidate their role in host shutoff. The work provides the foundations to further elucidate the mechanisms of action and biological relevance.

Results

Identification of VACV Post-Replicative Genes That Show the Ability to Induce Host Shutoff in a Preliminary Screening Utilizing *Gaussia* Luciferase Assay

To identify VACV post-replicative genes that contribute to virus-induced host shutoff, we co-transfected virus infected HeLa cells treated with cytosine arabinoside (AraC) with a post-

replicative viral gene and a EF1α-*Gaussia* luciferase reporter plasmid. AraC treatment inhibits viral DNA replication and blocks expression of post-replicative genes, which allows expression of only the VACVWR late gene that is co-transfected with EF1α-*Gaussia*. The post-replicative genes were constructed downstream of the intermediate viral promoter G8R, which allows for expression of the gene during VACV infection when AraC is present (see Figure 3.1-A). The benefit of this assay is that it can be utilized to screen the mechanisms of VACV viral late genes whose functions have not been identified or fully characterized. This viral gene was co-transfected with a reporter plasmid that has the constitutive human cellular elongation factor 1 alpha (EF1α) promoter inserted upstream of a *Gaussia* luciferase gene of a plasmid vector. We screened 54 VACV post-replicative genes—At the time of this work, our post replicative library contained 54 out of 93 VACV post replicative genes due to difficulty cloning (Yang et al., 2010; see Table 3.1), and eight genes showed a 70% or greater significant reduction in *Gaussia* luciferase activity including the decapping enzyme VACWR115, or D10L, a major contributor to VACV induced host shutoff by accelerating mRNA turnover (see Figure 3.1-B).

Table 3.1

VACWR	VACV COP	Relative *Gaussia* Luciferase Activities	*p*-value	Functional Category
008	C19L	0.79	ns	Truncated
020	C8L	1.09	ns	Unknown
025	C3L	0.86	ns	Host interaction
033	K2L	0.80	ns	Host interaction
048	F9L	0.98	ns	Virion Association
052	F13L	0.77	ns	Virion Association

056	F17R	1.84	**	Virion Association
063	E7R	1.61	*	Unknown
064	E8R	0.87	*	Virion Association
066	E10R	0.89	ns	Virion Association
067	E11R	1.90	*	Virion Association

VACWR	VACV COP	Relative *Gaussia* Luciferase Activities	*p*-value	Functional Category
069	O2L	0.88	ns	Unknown
069.5	O3L	1.08	ns	Virion Association
070	I1L	3.88	*	Virion Association
071	I2L	0.42	ns	Virion Association
074	I5L	0.20	**	Virion Association
075	I6L	2.94	*	Virion Association
079	G3L	0.49	ns	Virion Association
081	G4L	1.48	ns	Virion Association
084	G6R	0.88	ns	Host interaction
085	G7L	0.53	ns	Virion Association
091	L4R	1.63	ns	Transcription
092	L5R	0.37	ns	Virion Association
093	J1R	2.03	ns	Virion Association
097	J5L	0.26	***	Virion Association
099	H1L	0.50	*	Transcription
100	H2R	0.43	*	Virion Association
102	H4L	3.85	ns	Transcription
104	H6R	0.66	*	Transcription
105	H7R	1.33	ns	Virion Association
107	D2R	2.21	ns	Virion Association
108	D3R	1.43	ns	Virion Association
115	D10R	0.11	***	Host interaction

VACWR	VACV COP	Relative *Gaussia* Luciferase Activities	*p*-value	Functional Category
120	A2L	2.38	*	Transcription
121	A2.5L	3.07	ns	Virion Association
125	A6L	4.86	***	Virion Association
128	A9L	0.59	ns	Virion Association
132	A13L	0.39	ns	Virion Association
133	A14L	0.42	*	Virion Association
134A	A14.5L	1.09	ns	Virion Association
135	A15L	1.27	**	Virion Association
137	A17L	0.16	***	Virion Association
139	A19L	1.59	**	Unknown
140	A21L	0.51	*	Virion Association
142	A22R	1.14	ns	DNA Replication
150	A27L	0.78	ns	Virion Association
151	A28L	0.82	ns	Virion Association
153	A30L	0.44	**	Virion Association
153.5	A30.5L	0.71	ns	Unknown
157	A34R	0.26	**	Virion Association
162	A38L	0.22	***	Virion Association
163	A39R	0.26	***	Truncated
167	A42R	0.68	**	Virion Association
168	A43R	0.23	**	Host interaction

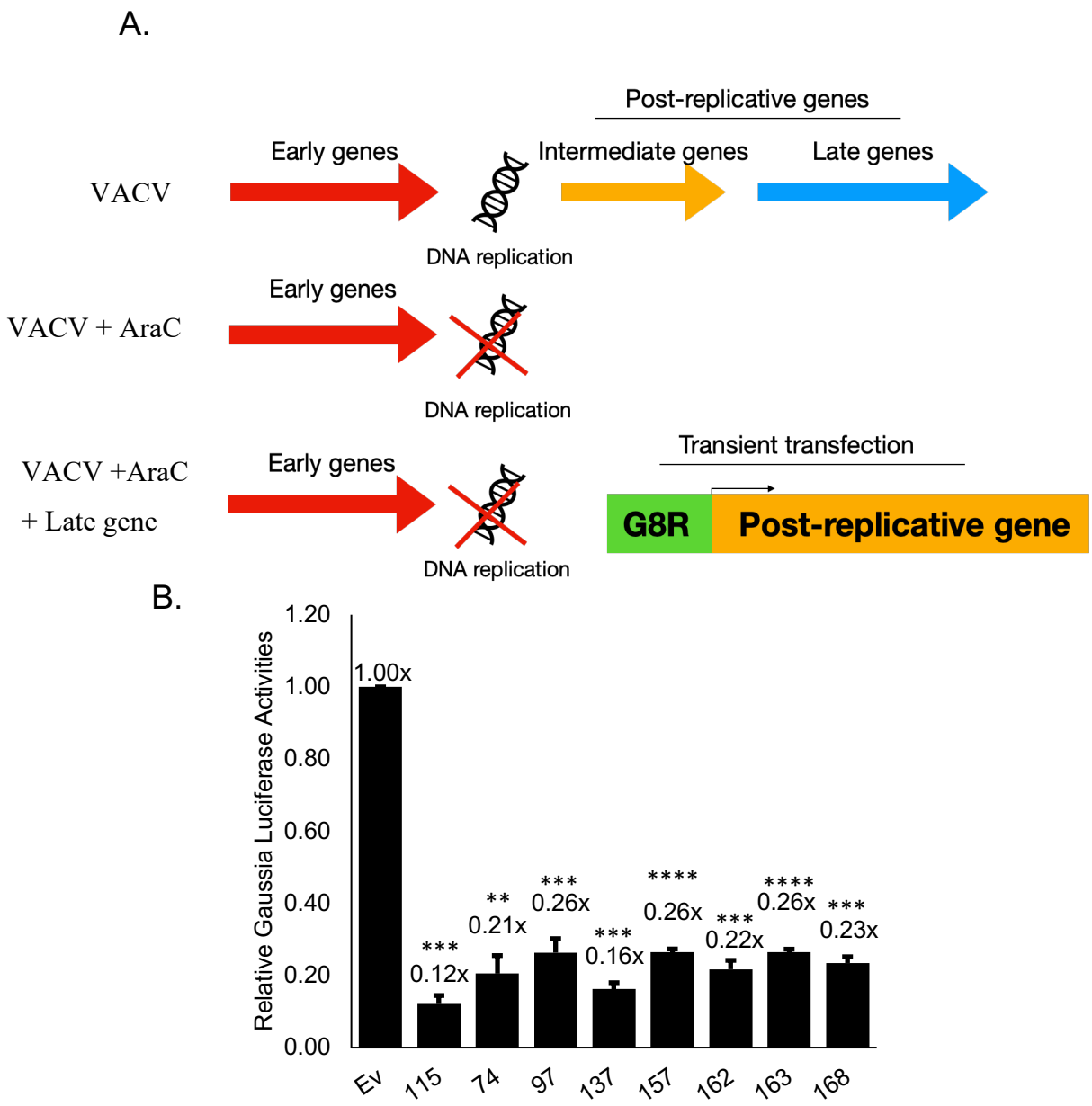
Screening Results of 54 Post-Replicative Genes in Infected HeLa Cells Treated With AraC and Utilizing Gaussia Luciferase Assay. Gaussia luciferase activities in media of cultured HeLa cells in a 24-well plate 24 h.p.i. with VACV at an moi of 5, treated with AraC (40mg/mL) , and co-transfected with EF1α-*Gaussia* luciferase reporter plasmid and designated VACVWR post-replicative gene. Significance was compared to mock. Error bars indicate the standard deviations of at least three replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

VACWR074 is conserved in chordopoxvirus and encodes for the ~9kDa protein I5 that is known to associate with the membranous components of assembling and mature virions. Deletion of I5 has been shown to be dispensable for viral replication in tissue culture (Unger et al., 2008). VACWR097 encodes for the ~16kDA protein J5 that is conserved in chordopoxvirus and entomopoxivurs and shares a C-terminal transmembrane domain homologous with G9 and A16. J5 has been shown to associate with entry fusion complex, and deletion of J5 results in reduced virus yield, suggesting it is essential for viral replication (Wolfe et al., 2012). VACWR137 encodes for the ~23kDa protein A17 that is involved in the early stages of virion assembly. A17 accumulates in the membranes of the rough endoplasmic reticulum and in the nuclear envelope, localizes to viral crescents and associates to the membranes of immature and intracellular mature virions. Deletion of A17 results in decreased virus yield (Rodríguez et al., 1996). VACWR157 encodes for the viral glycoprotein A34 that is present in the outer membrane of extracellular virus (EEV) and has been shown to play a role in the release of EEVs, EEV infectivity, and virus virulence. Deletion of A34 results in reduced plaque size indicating reduced infectivity (McIntosh & Smith, 1996). VACWR162 encodes for the ~33kDA integral membrane glycoprotein A38 with a sequence similarity to integrin-associated protein (IAP), which can explain the reasoning behind the influx of Ca2+ into cells and induction of cell necrosis along with reduced virus production and spread when A38 is overexpressed (Sanderson et al., 1996).

A38 is not essential for virus virulence as deletion of the gene yielded similar production of IMV and EEV in tissue culture and murine intranasal model (Sanderson et al., 1996). VACWR163 encode for the viral protein A39, a member of the semaphoring family involved in neuronal development. Some members of this family function immunologically, which is supported by the fact A39 can elicit cytokine production from monocytes (Comeau et al., 1998). VACWR168 encodes for the glycosylated transmembrane protein A43 that conserved in orthopoxviruses. A43 has been shown to localize with the Golgi, and deletion of A43 did not show reduction in growth curves compared to WT, suggesting its nonessential for viral replication in tissue culture (Sood & Moss, 2010).

These results expand our knowledge on the functions of these viral genes on virulence and identifies previously unknown host shutoff factors that require further investigations to elucidate their mechanism of action.

Figure 3.1



Screening of VACV Post-Replicative Genes in Infected HeLa Cells Treated With AraC and Utilizing Gaussia Luciferase Assay . (A) Schematic representing the VACV post-replicative gene screening in infected cells treated with AraC to inhibit DNA replication, and co-transfected with EF1α-*Gaussia* luciferase reporter plasmid. (B) *Gaussia* luciferase activities in media of cultured HeLa cells in a 24-well plate 24 h.p.i. with VACV at an moi of 5, treated with AraC (40mg/mL) , and co-transfected with EF1α-*Gaussia* luciferase reporter plasmid and designated VACVWR post-replicative gene. Significance was compared to mock. Error bars indicate the standard deviations of at least three replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

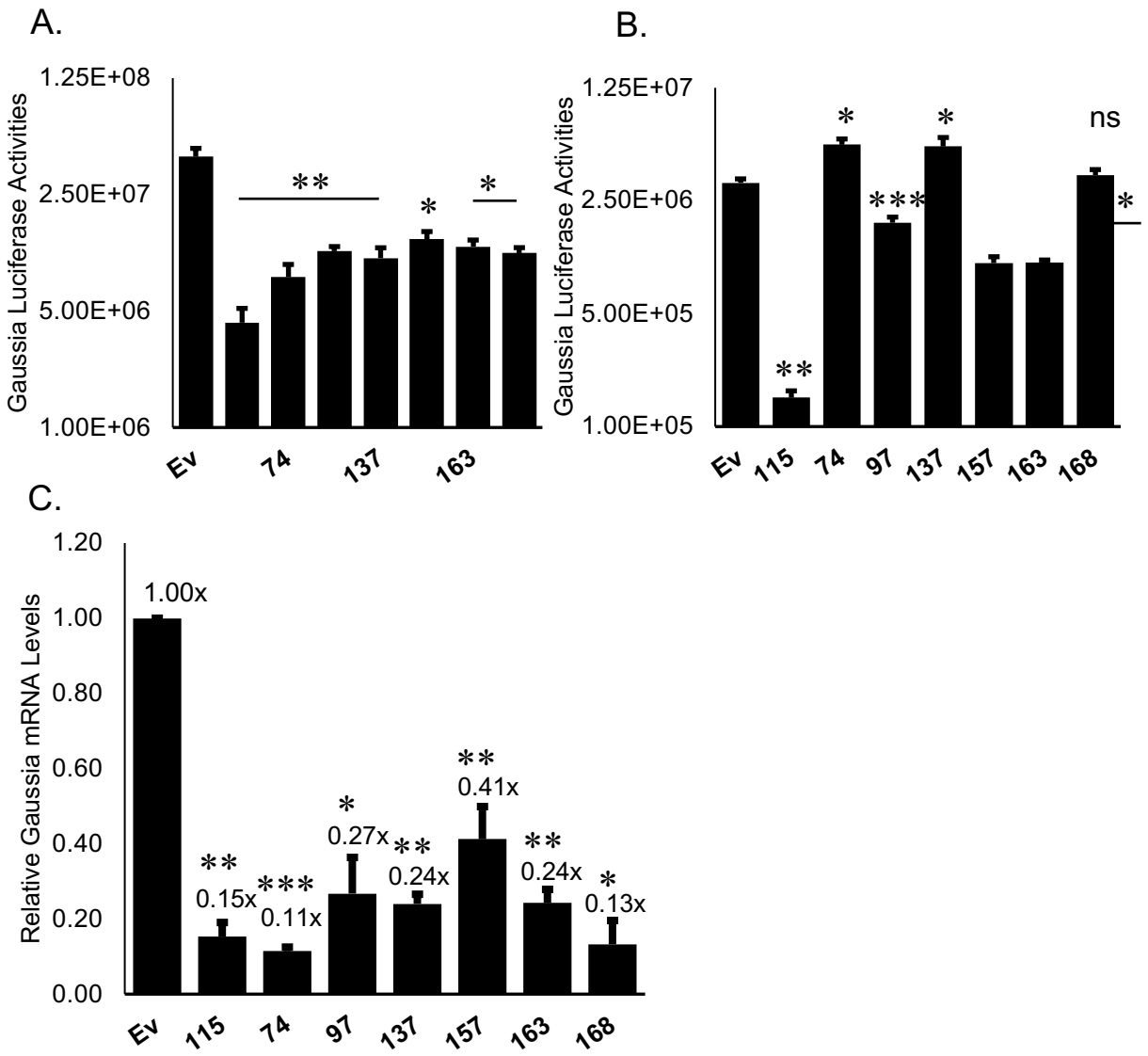
Post-Replicative Genes Show a Reduction in Total *Gaussia* Luciferase Activities and Reduced *Gaussia* mRNA Levels

We wanted to further identify the mechanisms the post-replicative genes had on host shutoff by comparing the expression of *Gaussia* luciferase in the media and lysate. This rules out the possibility the Gaussia luciferase is being retained within the cell. Virus infected HeLa cells treated with AraC 1 h.p.i. in order to inhibit expression of post replicative genes were co-transfection with seven of the post-replicative viral genes that showed a strong reduction in expression of the *Gaussia* luciferase activities. At 24 h.p.i. the media and lysate were collected and analyzed using *Gaussia* luciferase assay. Consistent with previous findings (Molina & Yang, 2022), a majority of the *Gaussia* luciferase activities (~99%) is located in the media. While all of the post-replicative genes showed a reduction in *Gaussia* luciferase in the media and overall (see Figure 3.2-A), only four showed a decrease in intracellular *Gaussia* luciferase (VACWR115 showed a 21-fold, VACWR097 showed a 1.7-fold, VACWR157 showed a 3.14-fold, and VACWR163 showed a 3.12-fold reduction) while the other three showed change or even an increase (VACWR074 showed a 0.58 fold increase, VACWR137 showed a 0.6-fold increase, and VACWR168 no significant difference; see Figure 3.2-B). These results confirm that these seven post-replicative genes show reduced *Gaussia* luciferase expression during the screening, and that a majority (~99%) of the *Gaussia* luciferase activity is found in the media. Interestingly, three of the seven post-replicative genes showed increase or no significant change in the intracellular *Gaussia* luciferase activities, which could suggest that alterations in post-translational processes such as protein trafficking may contribute to virus induced host shutoff. However, the reduction of *Gaussia* luciferase activities were mostly due to a total reduction of

Gaussia luciferase expression, but not because the *Gaussia* luciferase activities were retained in the cells.

To further characterize the reduction in *Gaussia* luciferase activities, the post-replicative screening was performed and *Gaussia* mRNA levels were measured utilizing quantitative real-time PCR (qRT-PCR). All seven post-replicative genes tested showed a significant decrease in *Gaussia* mRNA levels when compared to empty vector, suggesting that the post-replicative genes contribution to host shutoff involving the downregulation of mRNAs (see Figure 3.2-C). These experiments provide insights into possible viral mechanisms that contribute to host shutoff and further investigations are required to determine the exact function of individual genes.

Figure 3.2

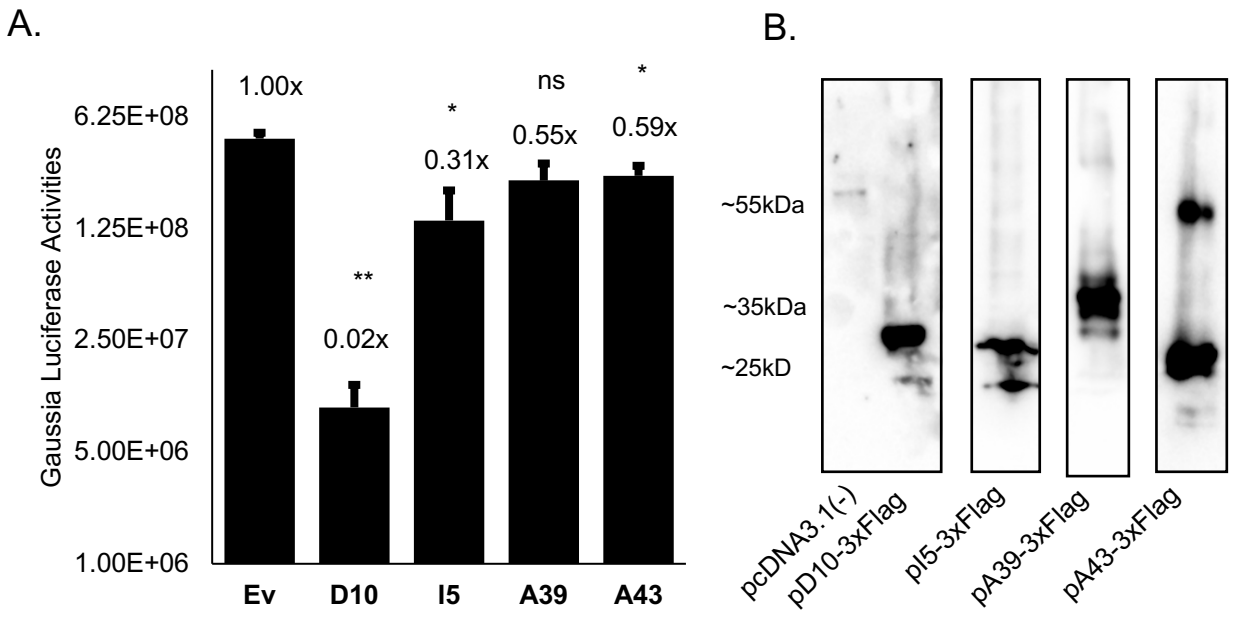


Comparing Gaussia Luciferase Activities in the Media and Lysate Shows an Overall Reduction in Gaussia Luciferase Activities and qRT-PCR Shows a Reduction of Gaussia mRNA Levels of the Post Replicative Gene Screening. Gaussia luciferase activities in (A) media and (B) cell lysates of the cultured HeLa cells during the VACV post-replicative gene screening. (C) *Gaussia* mRNA levels from isolate total RNA from cultured HeLa cells during the VACV post-replicative gene screening. Used as a normalization factor for different samples was 18S rRNA. Error bars indicate the standard deviations of at least three replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

I5 and A43 Reduce *Gaussia* Expression in a Dose Dependent Manner

To determine if the post-replicative genes could reduce *Gaussia* expression outside the context of the screening, codon-optimized plasmids of VACWR115 (D10L), VACWR074 (I5L), VACWR163 (A39R), and VACWR168 (A43R) containing a C-terminal 3x Flag tag were co-transfected with EF1α-*Gaussia* luciferase reporter plasmid into HeLa cells. The reduction in *Gaussia* luciferase activities by the post-replicative genes were found to be dose dependent, and a 5:1 ratio (post-replicative gene: EF1α-*Gaussia* reporter plasmid) of D10 showed a 47-fold reduction in *Gaussia* luciferase activity, and I5 showed a 3.2-fold reduction in *Gaussia* luciferase activity, and A43 showed a 1.7-fold reduction in *Gaussia* luciferase activity (see Figure 3.3-A).

Figure 3.3



Post-Replicative Genes Reduce Gaussia Luciferase Expression in a Dose-Dependent Manner. (A) *Gaussia* luciferase activities of plasmids containing post-replicative genes co-transfected with EF1α-*Gaussia* luciferase reporter plasmid into cultured HeLa cells and cell lysate collected for *Gaussia* luciferase assay 24hr post transfection. (B) Cell lysate was collected from cultured HeLa cells transfected with plasmids containing post-replicative genes and processed for SDS-Page and Western blot analysis. Error bars indicate the standard deviations of at least three

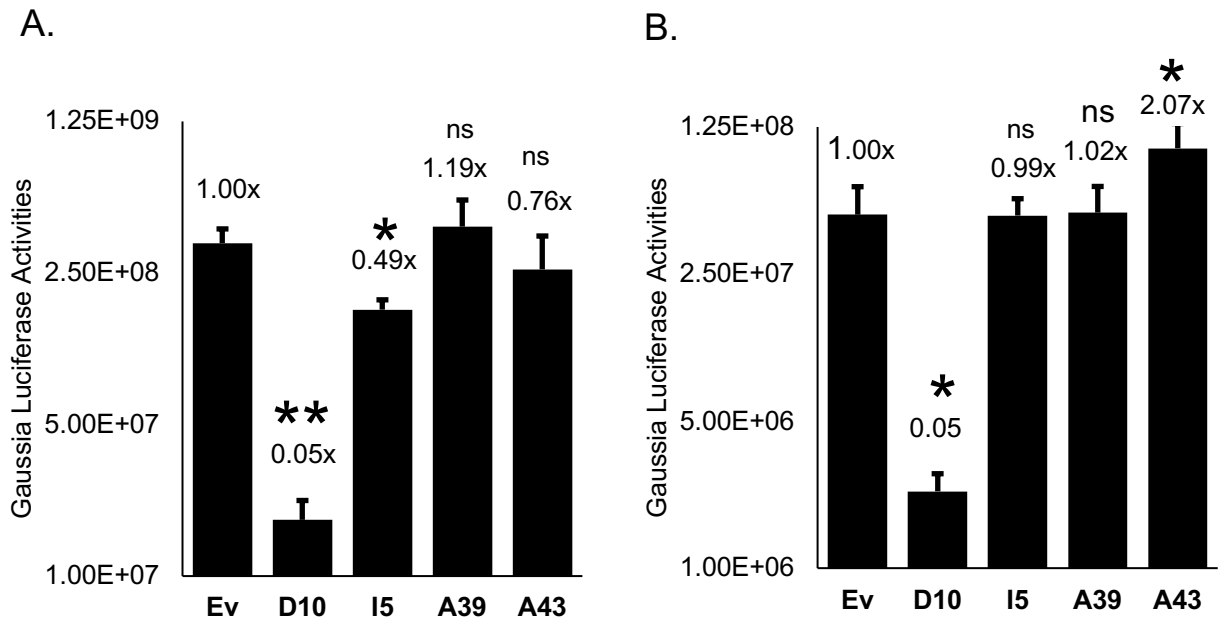
replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

Western blot analysis was performed to analyze the post-replicative gene expression in transient transfected HeLa cells. The bands were consistent with the expected MW of D10 (~31kDa), I5L (~11.5kDa), A39 (~36.4kDa), and A43 (~25.3kDa; see Figure 3.3-B).

To further characterize the reduction of *Gaussia* luciferase activity by plasmid containing VACV post-replicative genes, the media and lysate from co-transfected cells were collected and *Gaussia* luciferase assay performed to compare the intra- and extracellular *Gaussia* luciferase activity. D10 and I5 both show a reduction in *Gaussia* luciferase in the media and Total *Gaussia* luciferase as a majority of the *Gaussia* luciferase is found in the extracellular environment (~99%).

Interestingly, the intracellular *Gaussia* luciferase activity of D10 and I5 are comparable (see Figure 3.4), as D10 shows a similar reduction in both extracellular and intracellular *Gaussia* luciferase activity, but I5 intracellular *Gaussia* luciferase activity is similar to empty vector. This suggests that I5 may disrupt the trafficking and secretion of *Gaussia* luciferase, and this blockage may have downstream effects on translation and contribute to host shutoff.

Figure 3.4



I5 Reduces the Overall Gaussia Luciferase Activity and Extracellular Gaussia Luciferase Levels, But Not the Intracellular Levels. Gaussia luciferase activities in (A) media and (B) cell lysates of cultured HeLa cells co-transfected with codon optimized plasmids containing post-replicative genes and *Gaussia* luciferase reporter plasmid at a 1:1 ratio.

Discussion

While VACV host shutoff factors have been extensively researched, aspects of the mechanisms that contribute to host shutoff remain to be elucidated, and VACV proteins that inhibit host protein synthesis are not fully characterized. The most well studied VACV gene that induced host shutoff is the VACV decapping enzyme D10 that accelerates mRNA turnover and down regulated gene expression. Previous work by our lab (Molina & Yang, 2022) indicated strong inhibition of host protein synthesis post DNA replication, and while D10 is a major contributor to host shutoff, a reduction in protein synthesis was observed independent of D10.

In this study we wanted to investigate viral genes that are expressed post-replication for their ability to contribute to host shutoff. We established a method to screen VACV post-

replicative genes utilizing AraC treatment during infection to suppress DNA replication, thus inhibiting post-replicative gene expression, and transfecting plasmid containing post-replicative genes downstream of the viral G8R intermediate promoter. This allows expression of individual post-replicative genes to be further characterized. Fifty-four VACV post-replicative genes were screened in this study and six genes showed < .50x , 2 genes showed < .40x (see Table 3.1), and 8 genes showed < .30x in relative *Gaussia* luciferase activity compared to empty vector (Figure 3.1-B). All 8 post-replicative genes showed a reduction in *Gaussia* mRNA in the post-replicative screening (Figure 3.2-C).

To investigate the post-replicative genes outside the context of the post-replicative screening, plasmids containing codon optimized post-replicative genes D10, I5, A39, and A43 were co-transfected with *Gaussia* luciferase reporter plasmid, and D10, I5, and A43 were able to reduce *Gaussia* luciferase activity in a dose-dependent manner. We analyzed the intracellular and extracellular levels of *Gaussia* luciferase and interestingly we found that while D10 was capable of decreasing intracellular and extracellular *Gaussia* levels in a comparable fashion, I5 only showed a reduction in the extracellular *Gaussia* luciferase. I5 still showed a reduction in overall *Gaussia* luciferase activity, as most *Gaussia* luciferase is secreted into the extracellular environment. These results identify previously unknown VACV host shutoff factors, and potential targets for therapeutics and/or attenuated viruses for vaccine.

Future investigations should elucidate the mechanisms of the viral genes identified here at host shutoff factors. Determining cellular proteins that interact with the host shutoff factors would reveal how host shutoff factors induce suppression of protein synthesis. Also, future investigations

should determine the specificity of the host shutoff. All together, these avenues would significantly enhance our understanding of VACV host shutoff and potentially reveal novel mechanisms and pathways that viruses manipulate to suppress cellular translation.

Materials and Methods

Cells and Viruses

HeLa cells (ATCC CCL-2) were cultured in Dulbecco's minimal essential medium (DMEM; Quality Biological) supplemented with 10% fetal bovine serum (FBS; Peak Serum), 2 mM glutamine (Quality Biological), 100 U/ml of penicillin (Quality Biological), and 100 μg/ml streptomycin (Quality Biological). The cells were grown under 5% CO_2 at 37°C.
VACV Western Reserve (WR) strain (ATCC VR-1354) was utilized in this study and virus was prepared and titrated as described elsewhere (Cantu et al., 2020; Cotter et al., 1998; S-W. Liu et al., 2015). VACV infection was carried out using DMEM containing 2.5% FBS. Virus was sonicated and diluted according to desired MOI. Medium containing viruses was added to the cultured cells and incubated at 37°C for 1h and replaced with fresh medium.

Chemicals and Antibodies

Cytosine-1-β-D-arabinofuranoside (AraC, C1768) was purchased from Sigma-Aldrich. Lipofectamine 2000 Reagent (11668-019) was purchased from Invitrogen. Opti-MEM Reduced Serum Medium (31985-070) used in transfection was purchased from Thermo Fisher Scientific. Mouse a-Flag monoclonal antibody (used for Western blotting) was purchased from Sigma-Aldrich (catalog no. F3165).

Codon Optimization, Plasmid Construction, and Transfection

The plasmid vectors containing post-replicative genes under the control of the intermediate viral promoter G8R were designed and constructed using Zero Blunt TOPO PCR Cloning Kit from ThermoFisher (catalog # 450245) per manufacturers instruction. The plasmid vectors expressing post-replicative genes (pcDNA3.1-I5L-3xFlag, pcDNA3.1-A39R-3xFlag, pcDNA3.1-A43R-3xFlag) were codon optimized for expression in human cells and manufactured by GenScript based on previously described pcDNA3.1-D10-3xFlag (35 mBio; Y. Mohr et al., 2021). Plasmid transfections were carried out using Lipofectamine 2000 (Thermo Fisher Scientific; catalog no. 11668019) according to the manufacturer's instructions.

VACV Post-Replicative Gene Screening

Cultured HeLa cells in a 24-well plate were infected with VACV WR at an MOI of 5 in infectious media. Following 1h post infection, infectious media was replaced with infectious media containing AraC (40mg/mL) , and co-transfected with EF1α-*Gaussia* luciferase reporter plasmid and plasmid containing VACVWR post-replicative gene. We collected 24 h.p.i media and performed *Gaussia* luciferase activity assay.

Western Blot Analysis

Western blotting was performed as described previously (Pant et al., 2021). Briefly, the samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 2% bovine serum albumin (BSA) or 5% milk plus 1% BSA at room temperature for 1h and then incubated with primary antibodies in the same blocking buffer at

room temperature for 1h or at 4°C overnight. After washing with Tris-buffered saline with Tween 20 (TBST) three times, membranes were incubated with secondary antibodies at room temperature for 1h and washed three times with TBST. Before imaging, the membrane was developed using SuperSignal West Femto maximum-sensitivity substrate (Thermo Fisher Scientific; catalog no. 34094).

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Ambion), followed by purification using an Invitrogen PureLink RNA minikit (Thermo Fisher Scientific). The RNA was used to synthesize cDNA using a SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions and using random hexamer primers. Relative mRNA levels were quantified by the CFX96 real-time PCR instrument (Bio-Rad) with All-in-One 2x quantitative PCR (qPCR) mix (GeneCopoeia) and primers specific for indicated genes. We used 18S rRNA as a normalization factor for different samples.

***Gaussia* Luciferase Activity Assay**

Gaussia luciferase activities were measured using the Pierce *Gaussia* luciferase flash assay kit (Thermo Fisher Scientific, 16158) by a luminometer, according to the manufacturer's instructions.

Statistical Analysis

Student's *t*-test was performed to evaluate statistical differences from at least three replicates. The following symbols were used to indicate statistical significance: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; **** $p \leq 0.0001$.

Acknowledgment

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Conflict of Interests

The authors declare that there is no conflict of interests.

Chapter 4 - Characterization of the SARS-CoV-2 Host Shutoff Factor nsp1 and Identification of an Additional SARS-Cov-2 Host Shutoff Factors

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Abstract

Characterization of functions of the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genes, the cause of the respiratory illness Coronavirus disease 2019 (COVID-19) is required for the development of therapeutics and vaccines. To develop a better understanding of the virus and its pathogenesis, we set out to characterize the possible host shutoff factor nsp1 of SARS-CoV-2 and identified other viral genes that contribute to host shutoff. Utilizing *Gaussia* luciferase reporter we showed nsp1 of SARS-CoV and SARS-CoV-2 had the strongest reduction of reporter activities among the seven human coronaviruses. We then created plasmids containing R124A,K125A, K164A,H165A, and Δ122-130 mutant nsp1 and showed they reduced the ability of nsp1 to induce host shutoff, indicating the residues are critical to nsp1 SARS-CoV-2 host shutoff. Next, we screened all the SARS-CoV-2 viral genes for their ability to induce host shutoff and identified structural and accessory proteins S, E, M, and N as potential host shutoff factors. The SARS-CoV-2 viral proteinase nsp5, responsible for processing the mature viral replicase proteins, was also shown to induce host shutoff. Our data suggests nsp5 may have a broad impact on reduction of host protein abundance. Characterizing SARS-CoV-2 nsp1 has indicated that it may be a target for the creation of attenuated strains in the

development of improved vaccines, and a potential target for therapeutic drugs, as it has been shown to play a role in inhibiting the cellular immune response. Future studies should determine how much nsp5 contributes to host shutoff and identify the cellular substrates it cleaves.

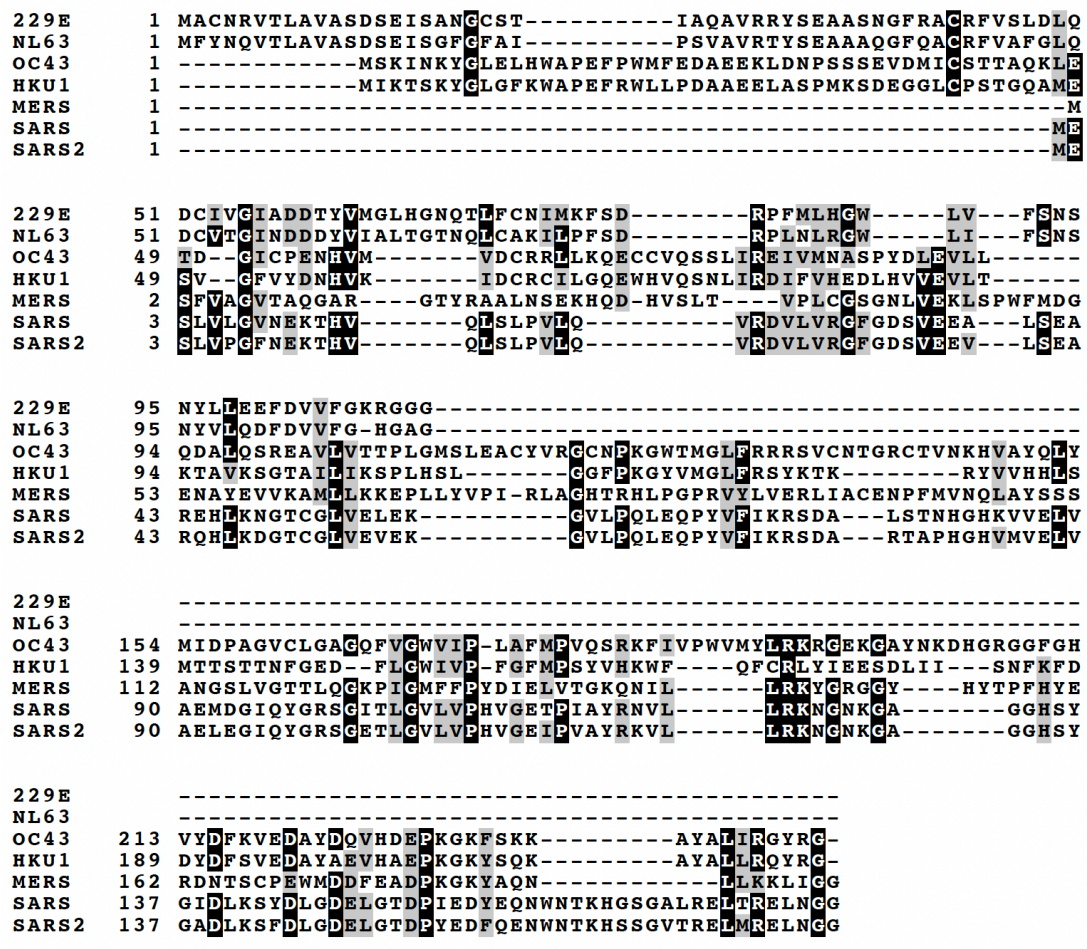
Keywords: SARS-CoV-2, Coronavirus, nsp1, host shutoff, translation control, *Gaussia* luciferase

Introduction

Over 81 million cases of Coronavirus disease 2019 (COVID-19) have been documented in the United States by the Centers for Disease Control and Prevention (CDC data tracker as of May 5, 2022) since the start of the current pandemic. This respiratory illness is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, a recent addition to the *Coronaviridae* family (Gorbalenya et al., 2020), Within the family *Coronaviriade*, SARS-CoV-2 belongs to the subfamily *Coronavirinae*, a group of enveloped, single-stranded positive sense RNA viruses that are further classified into four genera: alpha-, beta-, gamma-, and deltacoronavirus (Narayanan et al., 2015). The seven coronaviruses known to be pathogenic to humans (HCoVs) belong to the alphacoronavirus and betacoronavirus genus and include: alphacoronaviruses HCoV-229E and HCoV-NL63; and betacoronaviruses HCoV-OC43, HCoV-HKU1, MERS-CoV, SARS-CoV, and SARS-CoV-2. While pathogenic coronaviurses predominantly cause mild to moderate respiratory disease; MERS, SARS, and SARS-CoV-2 are known to cause severe respiratory illness (Beyer & Forero, 2022). Multiple sequence alignment shows SARS-CoV-2 shares a 79% sequence similarity with SARS-CoV and a ~50% sequence similarity with MERS-CoV (Rabaan et al., 2020). The most recent COVID-19 pandemic and the

threat of future pandemics has revealed the importance of studying the molecular mechanisms of coronavirus replication to improve current therapeutics and treatments.

Figure 4.1



HCoV nsp1 Alignment. Multiple sequence alignment of the seven HCoVs nsp1s were performed using T-Coffee and the figure was prepared using Boxshade (OnWorks®, 2022).

Like all viruses, coronaviruses depend on host translational machinery to synthesize viral proteins required for replication. To ensure efficient viral replication, viruses employ various mechanisms to interfere with host cell translation resulting in a global suppression of host gene expression, termed host shutoff (Dhungel et al., 2020). The most well studied host shutoff factor of HCoVs is the nonstructural protein 1 (nsp1), the N-terminal component of the polyprotein 1a (pp1a) that is found in both alpha- and beta-coronaviruses (Narayanan et al., 2015). SARS-CoV nsp1 utilizes a two-pronged approach to induce host shutoff by binding to the 40S ribosomal unit

thus inactivating its translational activity and promoting cellular mRNA degradation through recruitment of a cellular endonucleases (Kamitani et al., 2006, 2009; Narayanan et al., 2015). The nsp1 proteins of HCoVs have different strategies to induce host shutoff, as nsp1 of MERS-CoV has been shown to not tightly associate with the 40S ribosomal subunit and shows a different cellular distribution compared to SARS-CoV (Lokugamage et al., 2015). The nsp1 of SARS-CoV-2 shares an 84.44% sequence similarity to SARS-CoV and encodes some of the conserved regions previously identified to be crucial for gene function (see Figure 4.3-A). We hypothesized SARS-CoV-2 nsp1 may induce host shutoff and utilize similar mechanisms as SARS-CoV.

In this study we build off the foundational studies conducted on SARS-CoV and other coronaviruses and apply the knowledge learned to characterize the functions of SARS-CoV-2 nsp1. Using *Gaussia* luciferase reporter plasmid we show SARS-CoV-2 nsp1 is capable of suppressing host translation and can reduce the *Gaussia* luciferase activity to similar levels as SARS-CoV, the two strongest host shutoff factors of the HCoVs nsp1s. We next created mutations in the previously defined conserved regions of SARS-CoV-2 nsp1 (see Figure 4.2-B) and showed all mutations were capable of reducing SARS-CoV-2 nsp1's ability to induce host shutoff. These results suggest nsp1 of SARS-CoV-2 utilizes a similar strategy as nsp1 of SARS-CoV, which has been shown by recently published research articles (Shen et al., 2020; Thoms et al., 2020). Even though nsp1 is the most well studied coronavirus host shutoff factor, this does not rule out the possibility that other viral genes may contribute to SARS-CoV-2 induced host shutoff, as SARS-CoV-2 has been shown to encode multiple mechanisms to suppress cellular gene expression (Finkel et al., 2021). We also identified several structural and accessory viral

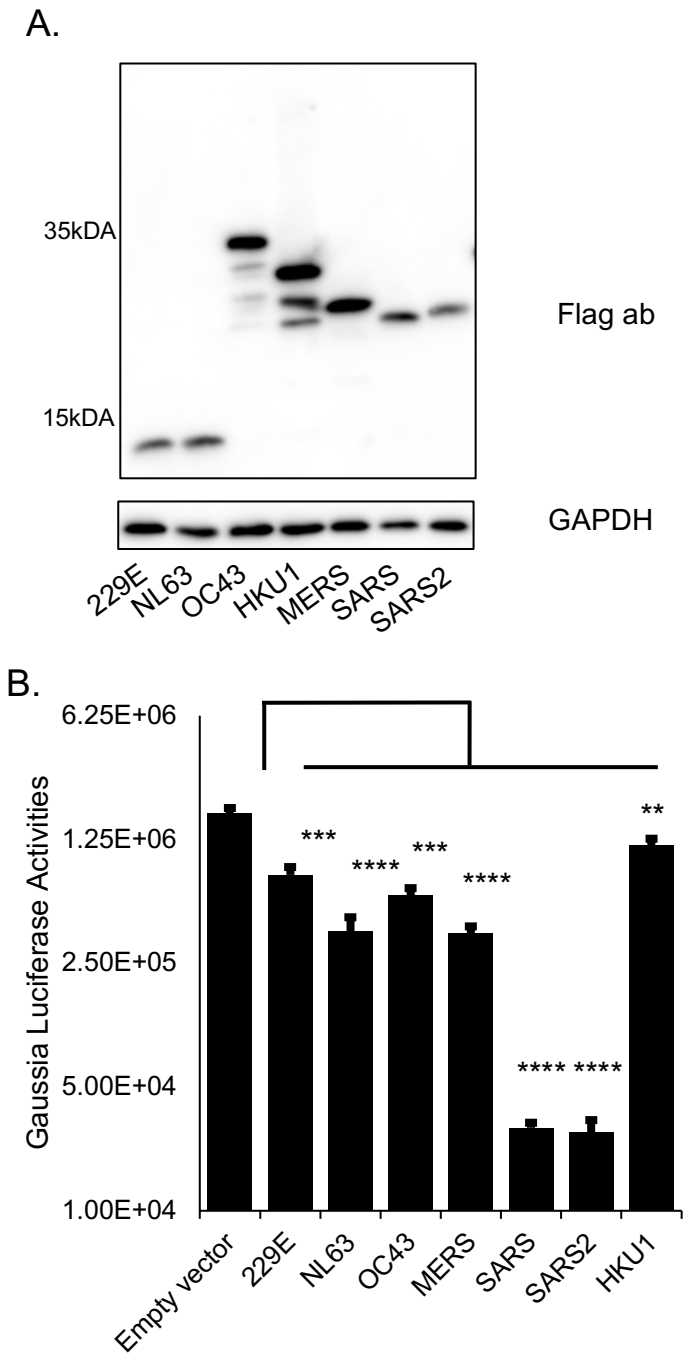
genes as well as nsp5 as capable of suppressing reporter protein synthesis as potential host shutoff factors. Taken together our results help us better understand the host interactions of SARS-CoV-2 genes that contribute to host shutoff and identify molecular mechanisms that need to be better understood to improve the therapeutics and treatments currently available for COVID-19.

Results

SARS-CoV-1 and SARS-CoV-2 nsp1 Proteins Are the Strongest Host Shutoff Factors Among Human CoVs

To determine if SARS-CoV-2 nsp1 could induce host shutoff and to compare it to other HCoVs nsp1s, we co-transfected 293T cells with plasmids containing HCoVs nsp1 containing a C-terminal flag tag and a *Gaussia* luciferase reporter plasmid previously described in Chapter 3. At 24h post-transfection, media was collected and analyzed using *Gaussia* luciferase assay. To test nsp1 protein expression, lysates were collected and processed for Western blot analysis. As expected, the two alpha-coronaviruses HCoV-229E and HCoV-NL63 bands appeared around ~9kDa, while the beta-coronavirus bands HCOV-OC43, HCoV-HKU1, MERS-COV, SARS-CoV and SARS-CoV-2 bands appeared near ~20kDa (see Figure 4.2-A). All the HCoVs nsp1s showed a significant reduction in *Gaussia* luciferase activities, with SARS-CoV and SARS-CoV-2 showing the greatest reduction with a fold change of ~58 and ~62 respectively (see Figure 4.2-B). These results suggest the nsp1 of SARS-CoV-2 can induce host shutoff with similar strength as SARS-COV nsp1.

Figure 4.2



SARS-CoV-1 and SARS-CoV-2 Show Similar Reduction Levels of Gaussia Luciferase Reporter Plasmid. (A) Cell lysate was collected from cultured 293T cells transfected with plasmids containing human coronavirus nsp1 for SDS-page and Western blot analysis. (B) *Gaussia*

luciferase activities of plasmids containing human coronavirus nsp1 genes co-transfected with *Gaussia* luciferase reporter plasmid into cultured 293T cells and cell lysate collected for *Gaussia* luciferase assay 24h post transfection. Error bars indicate the standard deviations of at least three replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$. The sequences for all seven HCoV nsp1s were obtained from GenBank and the respective GenBank accession number is provided (see Table 4.1).

Table 4.1

Human coronavirus	Sequence source
229E (alpha)	>NP_073549.1 replicase polyprotein 1ab [Human coronavirus 229E]
NL63 (alpha)	>YP_003766.2 replicase polyprotein 1ab [Human coronavirus NL63]
OC43 (beta)	>YP_009555246.1 leader protein [Human coronavirus OC43]
HKU1 (beta)	>YP_460018.1 nsp1 [Human coronavirus HKU1]
MERS (beta)	>YP_009047229.1 nsp1 protein [Middle East respiratory syndrome-related coronavirus]
SARS (beta)	>NP_828860.2 nsp1-pp1a/pp1ab [Severe acute respiratory syndrome-related coronavirus]
SARS2 (beta)	>YP_009725297.1 leader protein [Severe acute respiratory syndrome coronavirus 2]

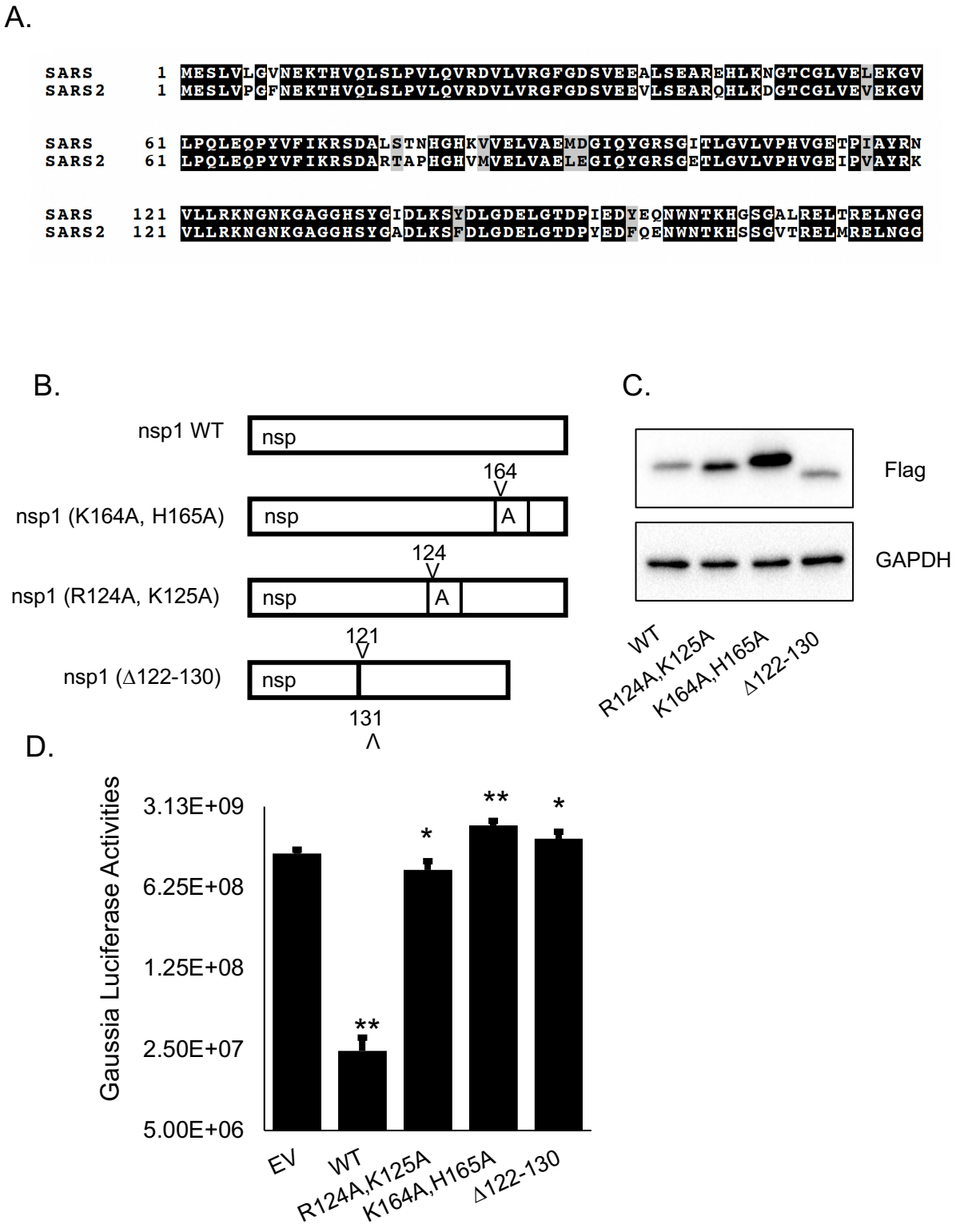
HCoV nsp1 Sequence Source

Mutations in SARS-CoV-2 Domains Previously Characterized in SARS-CoV nsp1 Show Reduction in the Ability to Induce Host Shutoff

Next, we performed an amino acid sequence alignment between SARS-CoV-2 and SARS-CoV and showed an 84.44% sequence similarity (see Figure 4.3-A). The shared amino

acid sequence similarity along with the similar levels of *Gaussia* luciferase activity reduction, we hypothesized the nsp1s from both viruses may induce shutoff by a similar mechanism. To test this hypothesis, we generated SARS-CoV-2 nsp1 plasmid mutants K164A,H165A and R124A,K125A (see Figure 4.3-B) that have previously been characterized for SARS-CoV. K164 and H165 amino acids are only conserved in SARS-CoV and SARS-CoV-2 nsp1 and alanine substitutions of these two residues inhibits nsp1 binding to the 40S ribosomal unit and reducing its ability to induce host shutoff (Narayanan et al., 2008). The R124 and K125 amino acid residues is part of a more conserved region found in all human betacoronaviruses nsp1s and alanine substitutions show these residues help the protein induce mRNA cleavage as the protein loses this ability when these residues are mutated (Lokugamage et al., 2012). We also created a deletion mutant (Δ122-130; see Figure 4.3-B) of the conserved region LLRKxGxKG that had been previously described to reduce the inhibition of luciferase expression as well as reduce the virulence in mice in MHV (L. Lei et al., 2013). To determine if the nsp1 of SARS-CoV-2 and SARS-CoV have similar characteristics, we co-transfected SARS-CoV-2 nsp1 WT and mutant plasmids into 293T cells along with a *Gaussia* luciferase reporter plasmid and collected the media 24 hrs post-transfection to perform *Gaussia* luciferase assay (see Figure 4.3-D). To test nsp1 mutant protein expression, lysates were collected and processed for Western blot analysis (see Figure 4.3-C).

Figure 4.3



Gaussia Luciferase Reporter Assay Shows Mutations in SARS-Cov-2 Nsp1 Reduce the Ability to Induce Host Shutoff. (A) Amino acid sequences of SARS-CoV-1 nsp1 (NP_828860.2) and SARS-CoV-2 nsp1 (YP_009725297.1) were aligned using NCBI Multiple Sequence Protein Blast. (B) Schematic of the SARS-CoV-2 nsp1 mutants in plasmid. (C) Cell lysate was collected

from cultured 293T cells transfected with plasmids containing mutated SARS-CoV-2 nsp1 for SDS-page and Western blot analysis. (D) *Gaussia* luciferase activities of plasmids containing mutated SARS-CoV-2 nsp1 genes co-transfected with EF1α-*Gaussia* luciferase reporter plasmid into cultured 293T cells and cell lysate collected for *Gaussia* luciferase assay 24hr post transfection. Error bars indicate the standard deviations of at least three replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

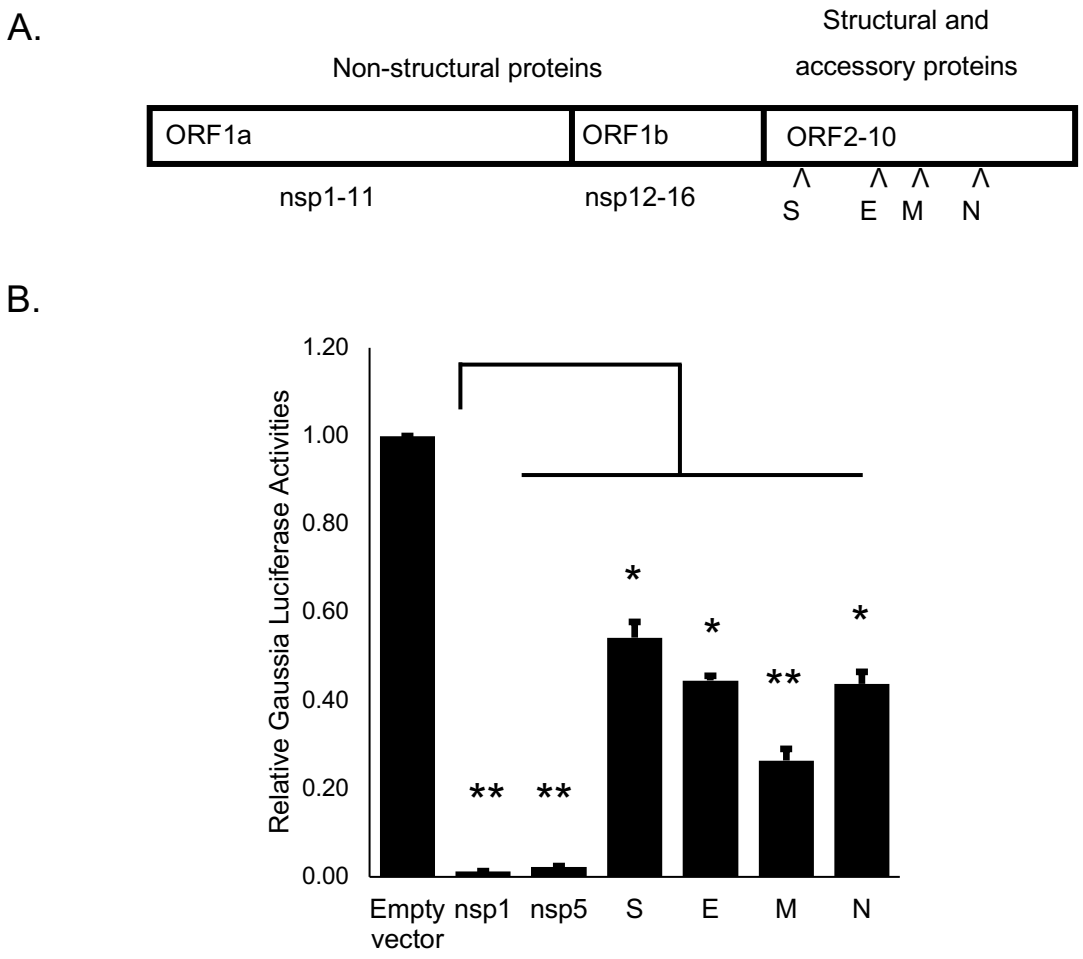
The results show the R124A,K125A nsp1 mutant retained its ability to reduce *Gaussia* luciferase activity, but this reduction was suppressed when compared to WT nsp1. The K164A,H165A nsp1 mutant and the Δ122-130 nsp1 mutant showed a complete inhibition of the proteins ability to induce host shutoff. These results suggest SARS-COV-2 nsp1 does share similar mechanism to induce host shutoff as SARS-CoV, as it has been shown that the K164 H165 residues of SARS-CoV are responsible for the protein binding to the 40S ribosomal unit, and alanine substitutions of these resides suppresses nsp1's ability to inhibit translation of cellular proteins, while the residues responsible for mRNA cleavage, R124 and K125, do not have as strong of a role as the 40S binding residues. Taken together, these results suggest the nsp1 protein of SARS-CoV-2 can inhibition protein synthesis, and that it may do so in a similar fashion.

Identification of Additional SARS-CoV-2 Shut Off Factors That Show the Ability to Induce Host Shutoff in Screening Utilizing *Gaussia* Luciferase Assay

Previous reports have shown other SARS-CoV genes may contribute to host shutoff during viral replication, and because SARS-CoV and SARS-CoV-2 viral genomes share ~79 sequence similarity, we hypothesized other SARS-CoV-2 viral genes may also contribute to the suppression of cellular protein synthesis. Upon viral infection, the SARS-CoV-2 viral genome is translated into two polypeptide precursors ORF1a and ORF1b. The precursors undergo

proteolytic processing by viral proteinases to generate the nonstructural proteins 1–16. The rest of the viral genome is made up of structural proteins and accessory proteins that are translated directly from the viral genome or viral subgenomic mRNAs (Nakagawa et al., 2016). The accessory proteins are encoded in individual ORFs 2-10, and the structural proteins are made up of the nucleocapsid protein (N), membrane protein (M), envelope protein (E), and the spike protein (S; see Figure 4.4-A). To screen the SARS-CoV-2 viral genes for their contribution to host shutoff, we co-transfected a plasmid containing each gene with a *Gaussia* luciferase plasmid reporter and collected the media after 24h for *Gaussia* luciferase assay. Nsp1 showed the highest reduction in *Gaussia* luciferase activity with a ~79 fold reduction (see Figure 4.4-B). Structural proteins S, E, M, and N showed a reduction in *Gaussia* luciferase activity with a 1.8-, 2.2-, 3.6-, and 2.2-fold change reduction respectively. Surprisingly nsp5 showed a ~44-fold reduction when co-transfected with the *Gaussia* luciferase reporter plasmid. Nsp5 is a viral proteinase and is responsible for processing the mature viral replicase proteins (Roe et al., 2021). Previous reports have shown nsp5 assists SARS-CoV-2 evade the host innate immune response by targeting RIG-1 and the mitochondrial antiviral signaling (MAVS) protein (Y. Liu et al., 2021). Our data suggests nsp5 may play a bigger role in induction of viral host shutoff, which is not an uncommon mechanism as other viral genes such poliovirus proteases 2Apro and 3Cpro, and HIV-1 PR target host proteins as well as their viral precursor substrates (Alvarez et al., 2006; Burgess et al., 2018).

Figure 4.4



Screening of SARS-CoV-2 Genes Utilizing Gaussia Luciferase Assay. (A) Schematic of SARS-CoV-2 genome with proteins annotated (B) *Gaussia* luciferase activities of plasmids containing SARS-CoV-2 genes co-transfected with EF1α-*Gaussia* luciferase reporter plasmid into cultured 293T cells and cell lysate collected for *Gaussia* luciferase assay 24hr post transfection. Error bars indicate the standard deviations of at least three replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

Table 4.2

SARS-CoV-2	% Identity to SARS-COV	Relative *Gaussia* Luciferase Activities	*p*-Value
Orf1ab	86.19		
nsp1	84.44	0.01	**
nsp2	68.34	0.51	*

nsp3	75.97		
nsp4	80.00	0.51	*
nsp5	96.08	0.02	**
nsp6	88.15	1.23	ns
nsp7	98.80	1.17	ns
nsp8	97.47	1.25	ns
nsp9	97.35	1.27	ns
nsp10	97.12		
nsp11		2.61	ns
nsp12	96.35	0.72	ns
nsp13	99.83	1.34	**
nsp14	95.07	3.72	*
nsp15	88.73	1.99	**
nsp16	93.29	1.61	**
S	75.76	0.54	*
Orf3a	72.36	1.40	*
E	94.74	0.45	*
M	96.00	0.26	**
Orf6	68.85	0.73	ns
Orf7a	85.25	1.12	ns
Orf7b	85.37	2.77	**
Orf8		0.81	ns
N	90.52	0.44	*
Orf10			

Host Shutoff Functional Screening of SARS-Cov-2 Proteins. The complete results from the preliminary screening of Figure 4.4. *Gaussia* luciferase activities of plasmids containing SARS-CoV-2 genes co-transfected with EF1α-*Gaussia* luciferase reporter plasmid into cultured 293T cells and cell lysate collected for *Gaussia* luciferase assay 24h post transfection. Error bars indicate the standard deviations of at least three replicates: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

Discussion

The ongoing COVID-19 pandemic and the risk of future outbreaks highlight the need to characterize SARS-CoV-2 interaction with the host and studying host shutoff can elucidate the mechanisms required for viral replication that can be used as a target to improve the currently available therapeutics and treatments for COVID-19. The most well studied human coronavirus host shutoff factor is nsp1, and fundamental research on SARS-CoV and other HCoVs have provided a foundation to understand the mechanisms of SARS-COV-2 viral replication. To elucidate whether nsp1 of SARS-CoV-2 could induce host shutoff, we used a EF1α-*Gaussia* luciferase reporter plasmid and showed SARS-CoV-2 could significantly reduce the *Gaussia* luciferase activity, and this reduction was comparable to SARS-CoV. We next performed an amino acid alignment of SARS-CoV and SARS-CoV-2 nsp1 and showed an 84.44% sequence similarity. This along with the similarity in *Gaussia* luciferase reduction, we hypothesized the genes may utilize similar mechanisms to suppress host cellular protein synthesis. To investigate the similarities between SARS-CoV and SARS-CoV-2 nsp1, we generated SARS-CoV-2 plasmids containing mutations to previously identified amino acid residues critical for the host shutoff function of SARS-CoV nsp1. These results showed the same amino acid residues involved in host shutoff for SARS-CoV nsp1 are also important for the host shutoff function of SARS-CoV-2 nsp1. These results suggest SARS-CoV and SARS-CoV-2 may utilize similar mechanisms to induce host shutoff.

Recently published papers characterizing the host shutoff of SARS-CoV-2 nsp1 are consistent with our findings. The lysine 164 located in the C-terminal was shown to be critical to binding of the 40S ribosome, and mutations at this residue inhibited the protein from interacting with the 40S ribosome and showed a reduction in inhibition of host protein synthesis (Shen et al., 2020). Structural analysis by cryo-electron microscopy revealed the C-terminal of nsp1 SARS-CoV-2 binds to and blocks the mRNA entry channel (Schubert et al., 2020; Thoms et al., 2020). The N-terminal R124 K125 and Δ122-130 residues were found to be critical for the stabilization of the C-terminal ribosome binding (Mendez et al., 2021). The 5' untranslated region of SARS-CoV-2 viral genomic and subgenomic mRNA was shown to stimulate translation of viral transcripts, and the R124 K125 residues along with R99 were found to be required for SARS-CoV-2 leader sequence mediated translation in the presence of nsp1 (Mendez et al., 2021; Schubert et al., 2020). Characterizing SARS-CoV-2 nsp1 has indicated that it may be a target for the creation of attenuated strains in the development of improved vaccines, and a potential target for therapeutic drugs, as it has been shown to play a role in inhibiting the cellular immune response (Beyer & Forero, 2022; de Lima Menezes et al., 2021).

To further characterize SARS-CoV-2 host shutoff factors, we performed a screening of the non-structural, structural, and accessory proteins to determine their effects to virus induced host shutoff. We also observed a reduction in *Gaussia* luciferase activities in the structural and accessory proteins S, E, M, and N. The SARS-CoV-2 viral proteinase nsp5 responsible for processing the mature viral replicase proteins showed a strong reduction in *Gaussia* luciferase activities. Previous reports have shown that nsp5 assists SARS-CoV-2 evade the host innate immune response by targeting RIG-1 and the mitochondrial antiviral signaling (MAVS) protein

(Y. Liu et al., 2021). Our data suggests nsp5 may have a broad impact on reduction of host protein abundance, which is not an uncommon mechanism for viral proteases as the poliovirus proteases 2Apro and 3Cpro, and HIV-1 PR target host proteins as well as their viral precursor substrates (Alvarez et al., 2006; I. Mohr, 2016). These results identified additional viral factors that may contribute to SARS-CoV-2 host shutoff, and that the viral proteinase nsp5 may target host cellular proteins along with its precursors viral protein substrates. More investigations into nsp5 will elucidate whether this viral proteinase targeting of host proteins is specific or broader than originally hypothesized. Elucidating additional host shutoff factors of SARS-CoV-2 and fully characterizing the viral mechanisms that contribute to efficient viral replication will provide a foundation for future viral pandemic research and improve the therapeutics and treatments for the current pandemic.

Materials and Methods

Cells and Viruses

293T cells were cultured in Dulbecco's minimal essential medium (DMEM; Quality Biological) supplemented with 10% fetal bovine serum (FBS; Peak Serum), 2 mM glutamine (Quality Biological), 100 U/ml of penicillin (Quality Biological), and 100 μg/ml streptomycin (Quality Biological). The cells were grown under 5% CO_2 at 37°C.

Chemicals and Antibodies

Lipofectamine 2000 Reagent (11668-019) was purchased from Invitrogen. Opti-MEM Reduced Serum Medium (31985-070) used in transfection was purchased from Thermo Fisher

Scientific. Mouse a-Flag monoclonal antibody (used for Western blotting) was purchased from Sigma-Aldrich (catalog no. F3165). Anti-DAPDH antibody (G-9) RP conjugated was purchased from Santa Cruz Biotechnology (sc-365062 HRP).

Codon Optimization, Plasmid Construction, and Transfection

The plasmid vectors expressing HCoV nsp1 (pcDNA3.1-HCoV-229E-3xFlag and pcDNA3.1-HCoV-NL63-3xFlag, pcDNA3.1-HCoV-OC43-3xFlag, pcDNA3.1-HCoV-HKU1-3xFlag, pcDNA3.1-MERS-CoV-3xFlag, pcDNA3.1-SARS-CoV-3xFlag, and pcDNA3.1-SARS-CoV-2-3xFlag) were codon optimized for expression in human cells and manufactured by GenScript based on previously described pcDNA3.1-D10-3xFlag (Cantu et al., 2020). The SARS-CoV-2 nsp1 substitution and deletion mutants were constructed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs , E0554S) per manufacturer instructions. The pCDH plasmids expressing SARS-CoV-2 genes were a gift from Pei-Hui Wang, Shandong University. Plasmid transfections were carried out using Lipofectamine 2000 (Thermo Fisher Scientific; catalog no. 11668019) according to the manufacturer's instructions.

Western Blot Analysis

Western blotting was performed as described previously (Pant et al., 2021). Briefly, the samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 2% bovine serum albumin (BSA) or 5% milk plus 1% BSA at room temperature for 1h and then incubated with primary antibodies in the same blocking buffer at room temperature for 1h or at 4°C overnight. After washing with Tris-buffered saline with

Tween 20 (TBST) three times, membranes were incubated with secondary antibodies at room temperature for 1h and washed three times with TBST. Before imaging, the membrane was developed using SuperSignal West Femto maximum-sensitivity substrate (Thermo Fisher Scientific; catalog no. 34094).

Gaussia Luciferase Activity Assay

Gaussia luciferase activities were measured using the Pierce *Gaussia* luciferase flash assay kit (Thermo Fisher Scientific, 16158) by a luminometer, according to the manufacturer's instructions.

Statistical Analysis

Student's *t*-test was performed to evaluate statistical differences from at least three replicates. The following symbols were used to indicate statistical significance: ns, $p > 0.05$; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $0.0001 < p \leq 0.001$; $0.001 < p \leq 0.0001$.

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Conflict of Interests

The authors declare that there is no conflict of interests.

Chapter 5 - Conclusion

The findings presented in this dissertation provide valuable tools that can be used to elucidate host shutoff mechanisms and identifies novel host shutoff factors that could lead to discovering new mechanisms involved in host shutoff. The results increase our understanding of viral host shutoff factors which may contribute to the development of better therapeutics as deletion of host shutoff factors have been shown to stimulate a more robust immune response during viral infection and result in viral attenuation (Burgess et al., 2018; Dunagan et al., 2021).

Previous methods to measure host shutoff in virus infected samples have extensively advanced the current field of virology. While some our considered the gold standard, they do contain limitations such as changing the cellular environment and being too labor consuming for rapid clinical testing. In Chapter 2 of this dissertation, we set out to develop a new high-throughput assay to analyze host shutoff by utilizing a cell line stably expressing *Gaussia* luciferase. The novel method allowed us to measure host shutoff in VACV infected cells quantitatively and in real-time from a single infected sample. We demonstrated the utility of *Gaussia* luciferase assay in VACV infected cells and showed the strongest inhibition of translation came from post-replication of the viral genome, which agreed with the current body of knowledge. The results indicated *Gaussia* luciferase is sensitive to changes involved in virus-host interactions, and this reporter could be used to study gene expression, promoter activity, and signal transduction in viral infection. This assay has practical application outside of viral infection, as inhibition and/or dysregulation of translation can be found in various pathologies such as cancer (Chu et al., 2016). Thus, the work done here contributes beyond the field of virology and has the potential to impact many areas of study.

Table 1.1 in Chapter 1 lists notable viruses that employ multiple mechanisms that contribute to host shutoff and demonstrates the complexity and interconnectivity of various cellular processes such as transcription, mRNA processing and export, and translation. This creates an opportunity for viral genes that act on other cellular processes to contribute to inhibition of translation. While multiple VACV factors have been suggested to contribute to host shutoff, how much they contribute and whether additional host shutoff factors exist has not been fully addressed. Our work in Chapter 2 suggests VACV encodes previously unidentified late gene viral factors contribute to host shutoff. In Chapter 3 of this dissertation, we developed a VACV late gene screening assay to identify additional VACV late genes that contribute to host shutoff. The benefit of this assay is it can be utilized to screen the mechanisms of VACV viral late genes other than host shutoff whose functions have not been identified or fully characterized. Our preliminary screening identified eight late genes that were capable of inducing host shutoff, a function not previously defined for these genes (Dhungel et al., 2020). One reason our results could differ from previous data is the novel use of the *Gaussia* luciferase assay to measure host shutoff. This study suggests disruption of post-translational processes such as protein trafficking may contribute to host shutoff. This raises two questions: What cellular proteins are impacted by disruption of protein processing and trafficking? and what is the mechanism responsible for inducing the host shutoff? The results identified in this dissertation could lead to the discover of new viral mechanism that contributes to host shutoff. We provide a unique method to measure host shutoff and its regulation by post-translational mechanisms such as trafficking, and future studies involving *Gaussia* luciferase could answer questions about its reliability as a reporter for the high-throughput screening assay to identify novel host shutoff factors.

Multiple vaccines and therapies have been developed to mitigate the risk of Coronavirus disease 2019, a respiratory illness caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A better understanding of the virus and its pathogenesis will allow for the development of improved treatments for the current and future coronavirus pandemics. As SARS-CoV-2 is a newly discovered virus, extensive research remains to characterize and understand how the virus interacts with the host cell. In Chapter 4, we set out to characterize the possible host shutoff factor nsp1 of SARS-CoV-2. The results showed nsp1 SARS-CoV-2 could induce host shutoff, and the conserved amino acid motifs are essential for this function. This work is consistent with the data of recently published articles and supports the use of the *Gaussia* luciferase reporter to measure host shutoff. The limitation of this work is these experiments were done with plasmid expression, future studies characterizing nsp1 within the context of viral infection will provide a truer picture of its contribution to viral replication. Characterizing SARS-CoV-2 nsp1 as a host shutoff factor identifies it as a target for the creation of attenuated strains in the development of improved vaccines, and a potential target for therapeutic drugs, as it has been shown to play a role in inhibiting the antiviral immune response (Beyer et al., 2020; de Lima Menezes et al., 2021).

Multiple viral genes of SARS-CoV-1 have been implicated in contributing to host shutoff. Whether or not additional viral genes encoded by SARS-CoV-2 contribute to host shutoff remains unknown. To identify additional SARS-CoV-2 host shutoff factors, we performed a screening of all the SARS-CoV-2 viral genes to measure their ability to induce host shutoff. The results suggest a few structural and accessory proteins are capable of inducing host

shutoff, as well as the viral proteinase nsp5 responsible for processing the mature viral replicase proteins (Roe et al., 2021). While previous data has suggested nsp5 target specific protein involved in the host innate immune responses, our data suggests nsp5 may have a broad impact on suppression of protein synthesis, which is not an uncommon mechanism for viral proteases as the poliovirus proteases 2Apro and 3Cpro, and HIV-1 PR target host proteins as well as their viral precursor substrates (Alvarez et al., 2006; I. Mohr, 2016). Two questions remain unanswered: What cellular proteins does nsp5 cleave? and Does this cleavage inhibit translation of newly synthesized proteins or alter the existing pool? Structural studies of nsp5 could identify protein domains involved in its cleavage and identify a new target for virus specific inhibitory drugs. As multiple viruses utilize viral proteases, a conserved protein domain provides the opportunity for broad-acting antiviral agents. This work identifies nsp5 as a potential host shutoff factor and future studies should characterize the underlying mechanism and identify its cellular substrates.

Together, the work in this dissertation furthers our understanding of how viruses interact with the host cell to induce host shutoff. Our results identify potential viral specific targets for the development of improved attenuated viruses for vaccines or oncolytic viruses. Future studies should elucidate the underlying mechanisms of the host shutoff factors, I5 of VACV and nsp5 of SARS-CoV-2, identified in this dissertation, and potentially identify unknown connections between translation regulation and various cellular processes such as those involved in the central dogma, the immune response, or metabolism. The continued development of the methods introduced in this dissertation as well as future technology and techniques utilized to characterize viral host shutoff will be beneficial to the field of virology, as well as various pathologies that

alter cellular translation, and to the field of cellular biology. All these avenues addressed in this dissertation will improve our understanding of how viruses interact with their host and allow us to better serve the public.

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Appendix – Summary of My Contributions

The following scientific article includes work done during my time in the Lee lab. My contributions include Figure 1B, Figure 5, and Figure S1. I also contributed to various revisions of the manuscript. I have permission to include this scientific article that I am a primary author on in the appendix.

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SCIENTIFIC REPORTS

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Autophagy–lysosome pathway alterations and alpha-synuclein up-regulation in the subtype of neuronal ceroid lipofuscinosis, CLN5 disease

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Neuronal ceroid lipofuscinoses (NCLs) are a group of inherited neurodegenerative lysosomal storage disorders. CLN5 deficiency causes a subtype of NCL, referred to as CLN5 disease. CLN5 is a soluble lysosomal protein with an unclear function in the cell. Increased levels of the autophagy marker protein LC3-II have been reported in several subtypes of NCLs. In this report, we examine whether autophagy is altered in CLN5 disease. We found that the basal level of LC3-II was elevated in both CLN5 disease patient fibroblasts and CLN5-deficient HeLa cells. Further analysis using tandem fluorescent mRFP-GFP-LC3 showed the autophagy flux was increased. We found the alpha-synuclein (α-syn) gene *SNCA* was highly up-regulated in CLN5 disease patient fibroblasts. The aggregated form of α-syn is well known for its role in the pathogenicity of Parkinson's disease. Higher α-syn protein levels confirmed the *SNCA* up-regulation in both patient cells and CLN5 knockdown HeLa cells. Furthermore, α-syn was localized to the vicinity of lysosomes in CLN5 deficient cells, indicating it may have a lysosome-related function. Intriguingly, knocking down *SNCA* reversed lysosomal perinuclear clustering caused by CLN5 deficiency. These results suggest α-syn may affect lysosomal clustering in non-neuronal cells, similar to its role in presynaptic vesicles in neurons.

Neuronal ceroid lipofuscinoses (NCLs) are a group of progressive neurodegenerative lysosomal disorders that predominantly affect children[1,2]. There are thirteen genetically distinct subtypes of the NCLs that are named based on the genes in which the mutations have been identified[3]. Intriguingly, these genes encode a variety of unrelated proteins that are localized to various cellular compartments. Detrimental mutations in any of these genes cause the proteinaceous buildups of subunit C of the mitochondrial ATP synthase and/or saposin A and D in lysosomal compartments[4–6]. The similar phenotype associated with these mutations suggests that the NCL-related proteins are involved in a common cellular pathway or contribute to processes that are functionally linked, resulting in similar lysosomal dysfunction and waste accumulation.

Macroautophagy (hereafter referred to as autophagy) is part of the lysosomal degradation system. In contrast to the endocytic degradation pathway, the autophagy process brings intracellular material, such as long-lived cytosolic proteins and unwanted organelles, to lysosomes for disposal. The autophagy pathway is therefore inseparable from lysosome functions. Abnormal autophagy has been associated with several neurodegenerative diseases and lysosomal storage disorders[7–9]. In NCLs, an altered or impaired autophagy pathway has also been implicated. For example, higher basal levels of LC3-II, a marker for autophagosome formation, have been observed in murine models of various subtypes of NCL, including CLN2/TPP1[10], CLN3[11], CLN5[12], CLN6[13], CLN7[14], and CLN10/

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cathepsin D[15] diseases. On the other hand, reduced autophagy flux has been found in CLN5$^{-/-}$ and CLN6$^{-/-}$ ovine neural cultures[16]. This discrepancy of the latter study may be due to different cell types or animal models used in the studies.

In this report, we use CLN5-deficient NCL human patient skin fibroblasts and CLN5 knockdown (KD) HeLa cells to examine the autophagy-lysosome pathway. The CLN5 gene encodes a lysosomal luminal glycoprotein[17,18]. The function of CLN5 in lysosomes remains elusive. A possible role in endosomal sorting was suggested for human CLN5[19]. Another report suggests a CLN5 orthologue in *Dictyostelium* has glycoside hydrolase activity[20]. Here we show in CLN5-deficient cells the basal level of LC3-II is elevated, the autophagy flux is increased, and the expression level of α-syn gene *SNCA* is up-regulated. α-syn is highly expressed in presynaptic neurons and primarily localized to synaptic vesicles[21,22]. The presence of cytoplasmic inclusions filled with insoluble α-syn aggregates is a hallmark of Parkinson's disease[23]. While α-syn has been implicated in several cellular processes, including synaptic vesicle endocytosis[24] and exocytosis[25], its exact function remains unclear. Despite being primarily associated with neurodegenerative disorders, both CLN5 and α-syn can be detected in a variety of tissues and cell types[26–31]. While this indicates more general roles of CLN5 and α-syn in non-neuronal tissues, there have been few studies performed to investigate these roles. The exogenously overexpressed α-syn has been shown to indirectly affect autophagy through the early secretory pathway protein Rab1a in cell culture systems[32]. Interestingly, we found the endogenous α-syn localizes to the lysosomes in human fibroblasts and HeLa cells. Furthermore, we uncovered a potential role for α-syn in regulating lysosomal positioning.

Results

Autophagy flux is increased in CLN5-deficient cells. As an initial step to examine whether the autophagy process might be altered with CLN5 deficiency, we compared the basal levels of an autophagy marker, LC3-II, in fibroblasts from control healthy individuals and fibroblasts derived from CLN5-deficient patients. LC3-II is a lipid-modified form of LC3 that is produced during autophagosome formation and is a commonly used readout for autophagy[33,34]. We found the protein level of LC3-II in CLN5-deficient patient fibroblasts was higher than in control cells (Fig. 1A). To ensure the effects observed were solely due to CLN5 deficiency in patient cells, we generated a CLN5 stable KD cell line with shRNA expression (Fig. 1B). The CLN5 protein level was dramatically reduced in CLN5 stable KD cells. Similar to CLN5-deficient patient cells, an increased LC3-II level was also observed in CLN5 KD HeLa cells. This is consistent with previous studies in various subtypes of NCLs[11–15]. When cells were treated with chloroquine (CQ) to block lysosomal degradation, there was a further accumulation of LC3-II in both wildtype and CLN5KD cells (Fig. 1C). This indicates that the higher basal levels of LC3-II seen in CLN5-deficient cells was not because the lysosome was unable to degrade LC3-II, but actually there was more LC3-II produced[34].

Next, we examined the level of P62, an adaptor protein that links ubiquitinated cargo proteins to LC3-II. Upon autophagy activation, P62 is recruited to autophagosomes and eventually degraded in autolysosomes. On the other hand, accumulation of P62 upon autophagy activation (e.g. by starvation) indicates autophagy impairment. Therefore, the level of P62 is a commonly used measurement for autophagy flux[33]. Unlike LC3-II, we did not observe changes in the basal level of P62 in CLN5-deficient cells (Fig. 1A,B). Next, we followed P62 levels upon starvation to test if CLN5 deficiency blocks autophagic degradation (Fig. 1D,E). Within 1.5 h of starvation, P62 levels were increased in both WT and CLN5 KD HeLa cells (Fig. 1D), suggesting P62 was up-regulated[35]. We then followed long term starvation for up to 8 hours. Starvation was performed in the presence of cycloheximide (to block protein synthesis) and bortezomib (to block proteasome degradation). Under these conditions, P62 was degraded equally efficient in WT and CLN5 KD cells, indicating CLN5 deficient cells were capable of degrading autolysosome materials (Fig. 1E). Similar results were found in control and patient fibroblasts (Fig. S1).

To further monitor the autophagy flux, we expressed mRFP-GFP-LC3 in human fibroblasts. The tandem fluorescent mRFP-GFP-LC3 has been used to follow the maturation progression of autolysosomes from autophagosomes, as the GFP fluorescence will be quenched in a more acidic environment such as autolysosomes, leaving the protein with only the mRFP signal[36]. Compared to control cells, the mRFP-GFP-LC3 emitted a stronger red fluorescent signal in CLN5-deficient cells (Fig. 2A). In addition, there were more punctate structures with only red fluorescence signals in CLN5 patient cells, suggesting more autolysosomes were present. The Mander's coefficient was shown as a percentage of red fluorescent signal overlapping with green fluorescent signal (Fig. 2A, right). In patient cells the RFP signal was less overlapped with GFP signal. As control experiments, we also treated control fibroblasts with either lysosome inhibitors or starvation to examine the mRFP-GFP-LC3 response (Fig. 2B). In cells incubated with CQ or bafilomycin A (baf A), the overlaps between mRFP and GFP increased to ~80% (Fig. 2B), compared to no treatment of ~50% (Fig. 2A, control). On the other hand, when autophagy was induced by starvation (HBSS), the overlaps between mRFP and GFP was reduced to ~30%, similar to what we observed in patient cells. This supports our notion that cells lacking the CLN5 protein have higher basal levels of autophagic activity.

Up-regulation of alpha-synuclein gene *SNCA* in CLN5-deficient cells. To further elucidate the changes of autophagy pathway in CLN5 disease patient cells, we performed quantitative PCR (qPCR) to follow a set of genes involved in the autophagy pathway (RT^2 Profiler PCR array from SABiosciences). Table 1 lists the genes that were greater than two-fold up or down-regulated (with a p value$<$ 0.05 from three repeats) in CLN5-deficient patient cells. Interestingly, none of the genes directly involved in autophagy initiation or machinery, e.g. *ATG* genes, were differentially expressed (data not shown). This suggests that the effects on autophagy flux with CLN5 deficiency are most likely through indirect regulation of the autophagy process. Among these genes, *SNCA* showed the greatest difference compared to control cells with ~90-fold increase in expression under normal cultured conditions. *SNCA* encodes α-syn, a protein well known for its role in the pathogenicity of Parkinson's disease[23].

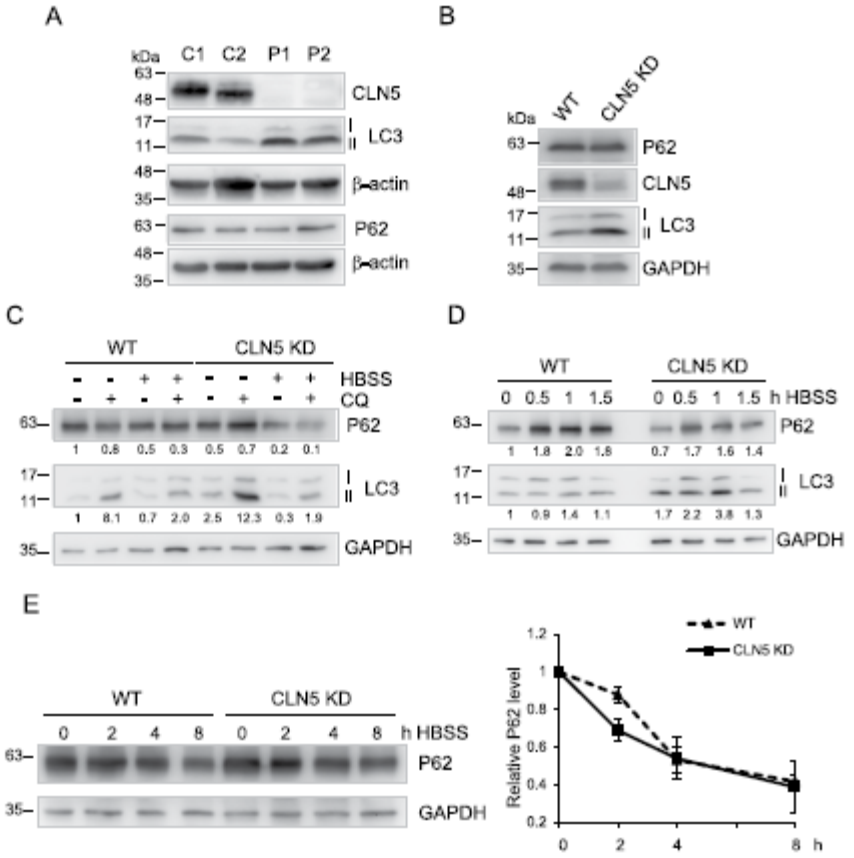

Figure 1. Autophagy is enhanced in CLN5-deficient cells. (**A**) Total lysates of two healthy control fibroblasts (C1 and C2) and two CLN5 disease patient fibroblasts (P1 and P2) were analyzed by immunoblotting. Basal levels of autophagic markers LC3-II and P62 were shown. The CLN5 was absent in patient cells. (**B**) Total lysates of WT and stable CLN5 KD HeLa cells were analyzed by immunoblotting. The CLN5 was greatly reduced in CLN5 KD cells. Basal levels of autophagic markers LC3-II and P62 were shown. (**C**) WT and Stable CLN5 KD HeLa cells were treated with CQ, HBSS, or HBSS + CQ for 4 h. Samples were analyzed by immunoblotting. The relative amounts of LC3-II and P62 after normalization with GAPDH are indicated. (**D**) WT and Stable CLN5 KD HeLa cells were incubated with HBSS for 0, 0.5, 1, 1.5 h. Samples were analyzed by immunoblotting. The relative amounts of LC3-II and P62 after normalization with GAPDH are indicated. (**E**) WT and Stable CLN5 KD HeLa cells were incubated with HBSS for 0, 2, 4, 8 h in the presence of cycloheximide and bortezomib. Samples were analyzed by immunoblotting. For degradation quantification on the right (N = 3), P62 was normalized with GAPDH signal in each lane. 0 h in each cell line was set as 1. Error bar represents SEM. β-actin and GAPDH were blotted as loading controls. All experiments were repeated at least three times.

Immunoblotting was performed to verify the qPCR results. In normal fibroblasts, α-syn was below the detection level. On the other hand, we were able to detect α-syn expression in CLN5 disease patient fibroblasts (Fig. 3A). Compared to control fibroblasts, HeLa cells synthesized a basal level of α-syn that was readily detectable. In CLN5 stable knockdown cells, α-syn expression level was further increased. Using immunofluorescence microscopy, we were able to visualize α-syn in CLN5-deficient cells (Fig. 3B). Consistent with immunoblotting results, the intensity of α-syn was greatly enhanced in cells lacking CLN5. Stably knocking down CLN5 in HeLa cells recapitulated the increased LC3-II and α-syn levels, indicating that the effects we observed in CLN5 patient fibroblasts were indeed due to CLN5 deficiency. Knocking down CLN5 by transient transfection in control fibroblasts as well as SH-SY5Y cells, however, did not increase the level of α-syn (Fig. S2), suggesting that the effects we observed in CLN5 disease patient cells and CLN5 stable knockdown cells are a consequence of long-term deficiency of CLN5.

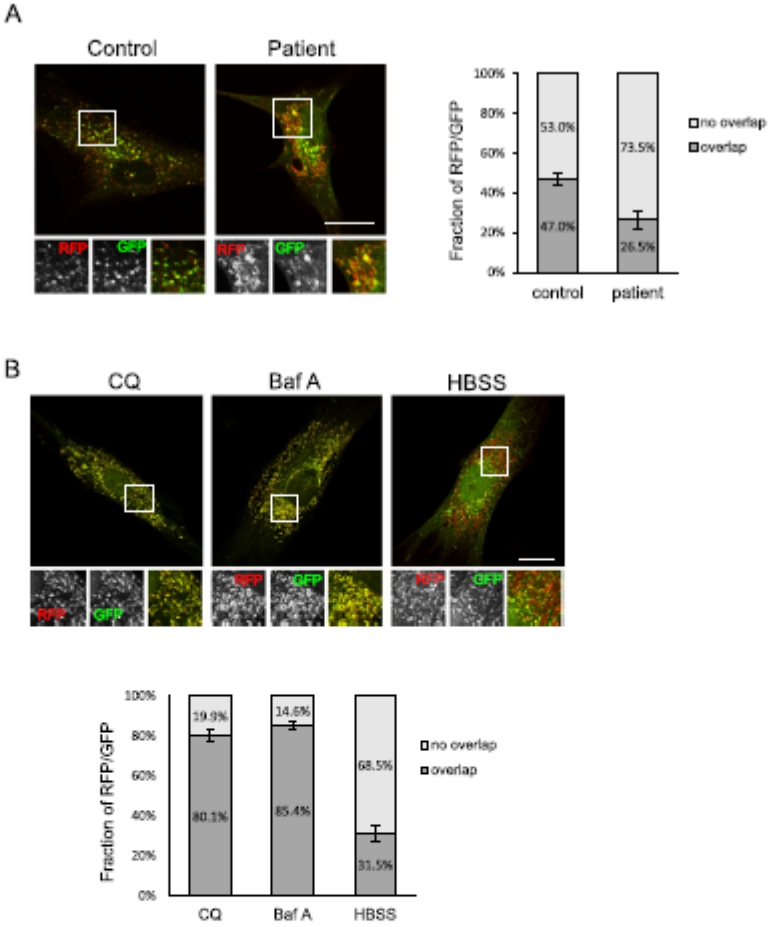

Figure 2. More autolysosomes are present in CLN5-deficient patient fibroblasts. (**A**) Control or CLN5-deficient fibroblasts that had been transfected with the mRFP-GFP-LC3 plasmid for 24 hours were fixed. GFP and RFP signal were visualized using confocal microscopy. The fraction of RFP/GFP signals that was overlapping was analyzed by the Mander's coefficient using the ImageJ JACoP plugin. In ImageJ, two channels of each image file were first split to two separate images. These two images were then used for plugin JACoP analysis. The threshold of each image was adjusted to reduce the background signals before performing JACoP Mander's coefficient. n = 15 cells, error bar represents SEM. (**B**) Control fibroblasts that had been transfected with the mRFP-GFP-LC3 plasmid for 24 hours were treated with CQ, baf A, or HBSS for 2 h prior to fixation. The fraction of RFP/GFP signals that was overlapping was analyzed by the Mander's coefficient using the ImageJ JACoP plugin. n = 7 cells, error bar represents SEM. All experiments were repeated at least three times. Scale bar: 20 μm.

α-syn is an abundant protein in presynaptic neuronal cells and is associated with synaptic vesicles[21,22]. However, it can also be detected in a variety of tissues[27–31]. Upon exogenous overexpression, α-syn has been reported to localize to several different compartments[37–40] and cause fragmentation of the Golgi apparatus[32,37]. Our finding of endogenously elevated expression levels of α-syn in CLN5-deficient cells prompted us to examine the localization of α-syn in these cells. We immunostained CLN5 KD cells with GRASP65 for the Golgi apparatus and Lamp1 for lysosomes, we found α-syn was largely colocalized with lysosomes but not the Golgi (Fig. 4A).

Several studies have suggested that α-syn can be degraded in the lysosomes via macroautophagy or the chaperone-mediated autophagy pathway[41–43]. Its accumulation has been observed in several lysosomal storage disorders (LSDs)[44–47]. Therefore, we subsequently analyzed if α-syn was localized in the lumen of the lysosomes as a substrate for degradation. In order to distinguish between outside and inside the lumen, we overexpressed GFP-Rab5 Q79L to induce enlarged endolysosomes[48]. Under normal conditions, endogenous α-syn was not

Gene Symbol	Fold Regulation	p-value
SNCA	+90.3923	0.0003
CTSS	+3.6365	0.0014
BCL2	−2.4529	0.0095
HGS	−2.3706	0.0065
CASP3	−2.3312	0.0008
CDKN1B	−2.1028	0.0002
TGFB1	−2.0955	0.0097
PTEN	−2.0499	0.0312

Table 1. Autophagy pathway qPCR results of CLN5-deficient patient fibroblasts. The genes with expression change (compared to control fibroblasts) >2-fold with a p-value < 0.05 are shown. Three independent qPCR were performed. +, up-regulated; −, down-regulated. The p values were calculated based on a Student's t-test of the replicate $2^{(-\Delta C t)}$ values for each gene in the control group and the CLN5 patient group.

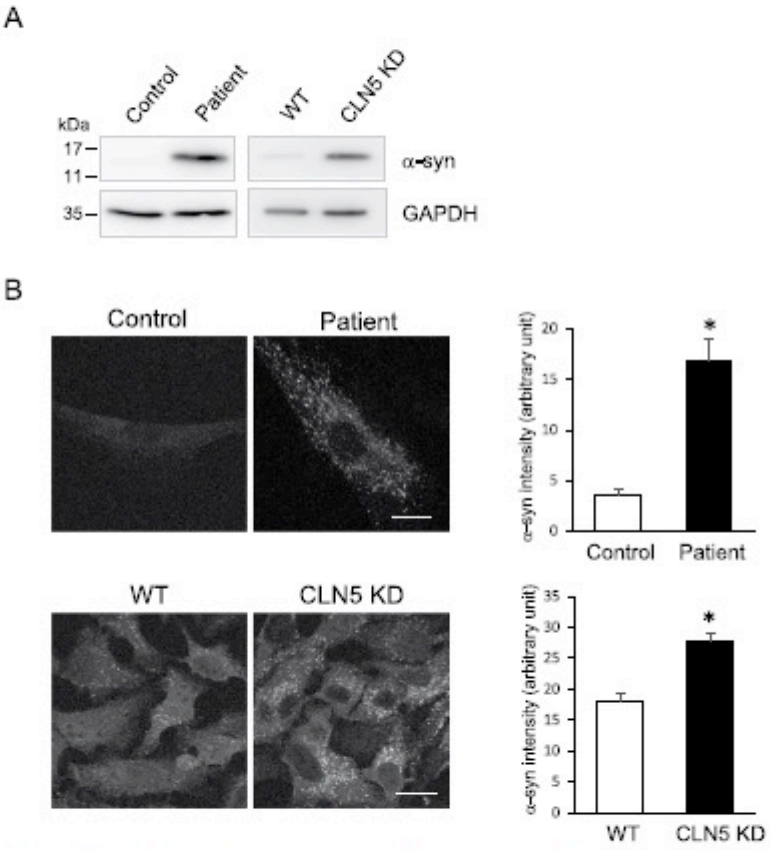

Figure 3. Higher levels of α-syn were detected in CLN5-deficient cells. (**A**) Total lysates from control and CLN5 disease patient fibroblasts, as well as WT and CLN5 KD HeLa cells were analyzed by immunoblotting. GAPDH was blotted as a loading control. (**B**) Control and CLN5 disease patient fibroblasts (top), as well as WT and CLN5 KD HeLa cells (bottom) were immunostained for α-syn. Signal intensity was quantified using ImageJ. Background was subtracted from the total intensity of each cell measured to obtain the correct total cell fluorescence. Data was analyzed using a Student's t test with two-samples assuming equal variances. *P < 0.0005, n = 5 cells for fibroblasts; *P < 0.0005, n = 20 cells for WT and CLN5 KD HeLa cells, error bar represents SEM. Images were acquired using confocal microscopy. Scale bar: 20 μm. All experiments were repeated at least three times.

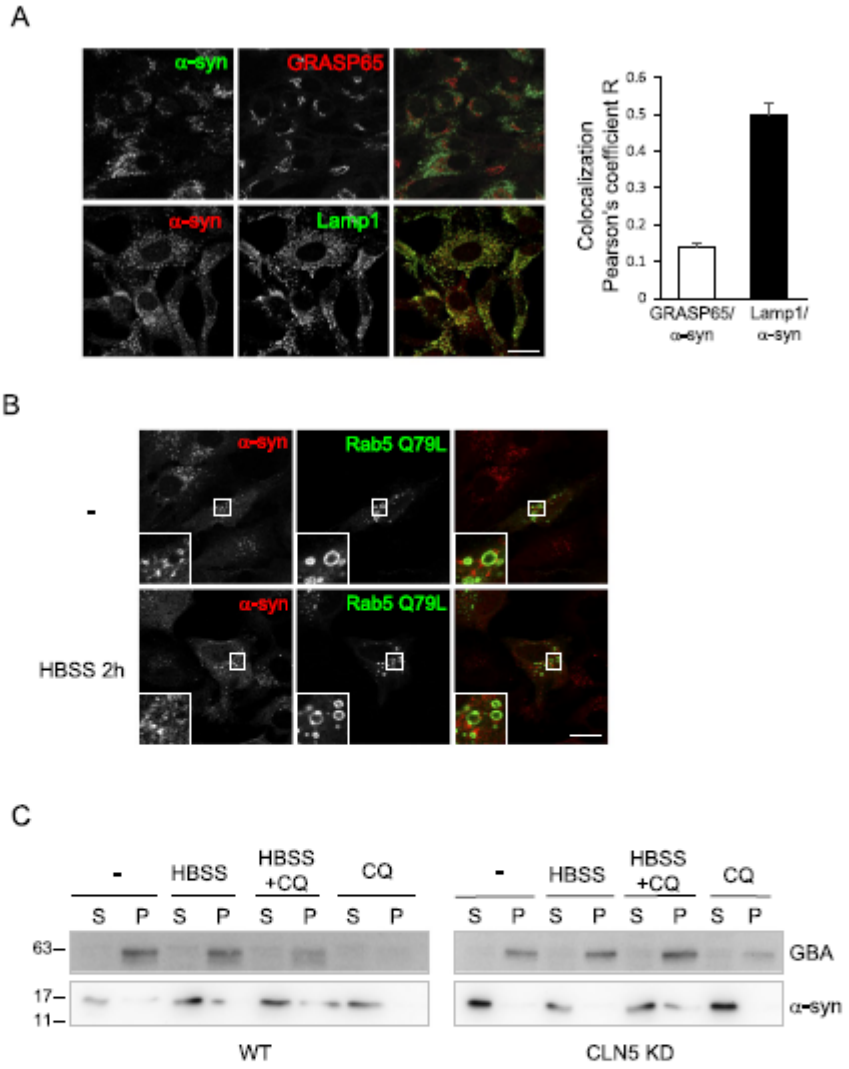

Figure 4. α-syn localizes to the vicinity of lysosomes. (**A**) CLN5 KD HeLa cells were fixed and immunostained for a Golgi marker, GRASP65 (top panel) and a lysosome marker, Lamp1 (bottom panel). The ImageJ JACoP plugin was used to analyze colocalization (Pearson's coefficient). In ImageJ, two channels of each image file were first split to two separate images. These two images were then used for plugin JACoP analysis. Five images in each set (total 59 cells in GRASP/α-syn set; total 42 cells in Lamp1/α-syn set) were analyzed. Error bar represents SEM. (**B**) α-syn does not localize to the lumen of lysosomes. CLN5 KD HeLa cells that had been transfected with the EGFP-Rab5 Q79L plasmid for 24 hours were incubated with HBSS for 2 h prior to fixation and immunostained for α-syn. In control (−), cells were left untreated. Cells were fixed and immunostained for CLN5. Images were acquired using confocal microscopy. Scale bar: 20 μm. (**C**) Subcellular fractionation of α-syn. WT and Stable CLN5 KD HeLa cells were treated with CQ, HBSS, or HBSS + CQ for 2 h. After fractionation, samples were analyzed by immunoblotting. S, soluble; P, pellet. All experiments were repeated at least three times.

present in the lumen (Fig. 4B, top), suggesting it is not subjected to degradation in lysosomes at the level of expression in CLN5-deficient cells. When cells were starved for 2 h to induce autophagy, no α-syn was found in the lumen of enlarged vacuoles either (Fig. 4B, bottom). Subcellular fractionation was performed to further

analyze whether α-syn was indeed cytosolic under these conditions (Fig. 4C). β-glucosylceramidase (GBA) is a lysosomal luminal protein and was found in the "P" fraction, indicating the lysosomes in the membrane fraction were intact. In both WT and CLN5 KD HeLa cells, α-syn was found primarily in the soluble cytosolic fraction under normal conditions with or without CQ, indicating α-syn was not subjected to degradation. Interestingly, when cells were starved with HBSS for 2 h, a small fraction of α-syn was detected in the pellet fractions of both cell lines. This suggests α-syn can be degraded in the lysosomes when autophagy is induced. This is consistent with previous findings that α-syn can be degraded in the lysosomes via macroautophagy or the chaperone-mediated autophagy pathway[41–43]. Furthermore, there was no difference between WT and CLN5 KD cells in α-syn subcellular distribution, suggesting that increased α-syn in CLN5 deficient cells was not because of dysfunctional lysosomes.

Alpha-synuclein and lysosomal clustering. The lysosomal localization of α-syn we observed reveals a possible function for α-syn in the lysosomal membrane. α-syn has a lipid binding property[49–51] and has been shown to cause clustering of synaptic vesicles[52]. Therefore, we examined whether α-syn has any role in the lysosomal membrane in CLN5-deficient cells where *SNCA* is upregulated. Under normal growing conditions, the lysosomes are distributed throughout the cytoplasm. However, in some LSDs the lysosomes become perinuclear[53,54]. In CLN5 KD cells, the lysosomes were clustered to the perinuclear region as well (Fig. 5A). Remarkably, when we knocked down *SNCA* in CLN5 KD cells, the lysosomes were redistributed to the periphery of the cell (Fig. 5A). Figure 5A α-syn panel and 5B show efficient knockdown of *SNCA* in CLN5 KD cells. The quantification analysis showed that, in CLN5 KD cells, the lysosomal distribution area returned to the WT level when α-syn was reduced by siRNA (Fig. 5C). This suggests that α-syn plays a role in lysosomal clustering, a property similar to that found in synaptic vesicles[52]. We also examined the LC3 and P62 levels when we knocked down *SNCA* in CLN5 KD cells (Fig. 5D). Unlike its effect on lysosome position, transient *SNCA* knockdown did not change LC3-II in CLN5 KD cells back to the levels in WT or SNCA knockout cells. This suggests that the increase of autophagy flux in CLN5 deficient cells is not due to *SNCA* up-regulation.

Discussion

Differential expression of α-syn has been shown in animal models of several NCL subtypes[47,55–58]. For example, accumulated α-syn has been found in CLN12 disease *Atp13a2*$^{-/-}$ mouse model and *Atp13a2* knockdown mouse primary neurons[47,55]. On the other hand, a lower level of α-syn protein was found in the motor cortex of a naturally occurring CLN5-deficient sheep[56]. In addition, some mouse model studies have examined *Snca* gene expression. *Snca* expression was found to be substantially reduced in the cortex of *Cln1*/*Cln5* double knockout mice[57], but not in *Cln1* or *Cln5* single knockout mice[57,58]. Yet in another study, *Snca* expression was highly up-regulated in the embryonic cortical neurons in *Cln1* knockout mice[58]. This highlights the complexity and variability of the regional and likely age-relevant expression.

In this study, we showed increased autophagy flux in CLN5-deficient cells by employing different measurements to monitor autophagy flux. In particular, the tandem fluorescent mRFP-GFP-LC3 assay demonstrated a higher basal autophagy flux in CLN5-deficient cells. The level of autophagy flux was similar to that of starvation induced autophagy in control cells. Since CLN5 disease is a lysosomal storage disorder, it is reasonable to assume increased autophagy is a compensatory effect in CLN5-deficient cells. Our data showed that lysosomal degradation of P62 was normal in CLN5-deficient cells, suggesting that CLN5 deficiency disrupts lysosome function other than lysosomal proteolysis.

Using PCR array of the autophagy pathway to analyze differential expression, we identified the *SNCA* gene as one of the autophagy-related genes up-regulated in CLN5 disease patient cells. Whether and how up-regulation of *SNCA* affects the autophagy process in CLN5-deficient cells remain to be determined. However, transient knocking down *SNCA* by siRNA in CLN5 KD cells did not reduce the LC3-II back to the level in WT cells, suggesting an indirect role of α-syn in regulating this process. Furthermore, while we observed up-regulation of the *SNCA* gene in CLN5 disease patient fibroblasts and stable CLN5 KD cells, transient CLN5 KD by siRNA in either fibroblasts or SH-SY5Y cells showed no up-regulation. These results suggest that *SNCA* up-regulation is a consequence of long-term CLN5 deficiency. A stable knockdown or knockout of CLN5 in SH-SY5Y cells or induced pluripotent stem cell (iPSC)-differentiated neuronal cells will help to elucidate whether *SNCA* up-regulation has a physiological relevance in NCL diseases.

The potential role of α-syn in lysosomal clustering we observed suggests a possible function of α-syn exists in non-neuronal cells. Using *in vitro* assays, it was shown that by binding to anionic lipid and synaptobrevin-2, a synaptic vesicle SNARE protein, α-syn caused clustering of the synaptobrevin-2 containing vesicles[25]. The clustering property of α-syn demonstrated herein is in agreement with the previous literature. For example, overexpression of α-syn inhibits synaptic vesicle release, possibly due to a reduced pool of readily releasable synaptic vesicles[60,61]. Similarly, deletion of α-syn increases neurotransmitter release[62]. It will be interesting to examine whether α-syn binds to any lysosomal SNARE proteins and whether the clustering effects of elevated levels of α-syn relate to the lysosome function in CLN5-deficient NCL and other NCLs in general.

Besides α-syn, defects in several proteins involved in endo-lysosomal trafficking, such as retromer subunit VPS35, leucine-rich repeat kinase 2 (LRRK2), Rab7L1, and Rab39B, are known to cause Parkinson's disease[63–68]. Supporting a role of α-syn in the endo-lysosome pathway, it has been documented that in cortical neurons α-syn localizes to the vicinity of endocytic compartments and interacts directly with VPS29, SNX1 (two retromer subunits), and Rab4A (an endosomal Rab GTPase)[69]. Whether our finding of α-syn affecting lysosome positioning in HeLa cells is physiologically relevant in neurons and contributes to pathogenicity of Parkinson's disease will need to be further examined.

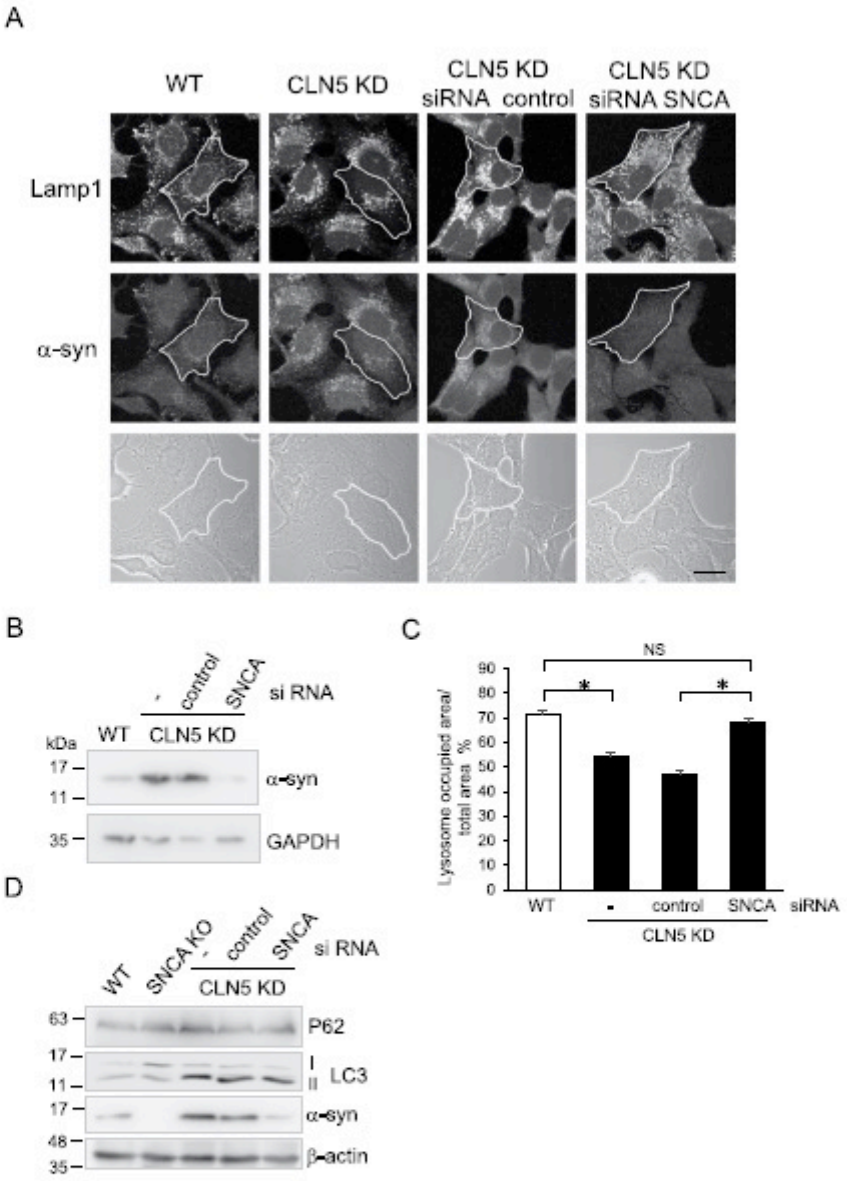

Figure 5. Knockdown of α-syn reverses the perinuclear lysosome clustering phenotype found in CLN5 KD HeLa cells. (**A**) HeLa or CLN5 KD cells were transfected with control or SNCA small interfering RNA (siRNA) for 48 hours prior to fixation. Lysosomes were visualized by immunostaining lamp1. Scale bar: 20 μm. (**B**) Immunoblot shows efficient knockdown of α-syn expression in CLN5 KD cells. GAPDH was blotted as a loading control. (**C**) The cellular distribution area of lysosomes was quantified in the conditions indicated. An outline of the cells was marked by tracing the cell border in phase contrast images. The total area of the cell and the area with lamp1 signal in the cell were measured using ImageJ "measure" function. One-way ANOVA followed by the Tukey's post hoc test was performed *$P < 0.05$, $n = 25$, error bar represents SEM. (**D**) Immunoblotting of WT, stable SNCA knock-out cell (SNCA KO), and siRNA of stable CLN5 KD as indicated. β-actin was blotted as a loading control. All experiments were repeated at least three times.

Materials and Methods

Cell culture and transfections. Cell culture media and reagents were purchased from Gibco and Hyclone. Healthy control human fibroblasts were purchased from Coriell Cell Repositories (C1: GM05757 and C2: GM00498). CLN5 disease patient fibroblasts (P1: c.671G > A, p. Trp224X and exon 4 deletion; P2: homozygous c.694C > T, p. Glm232X) were received from Massachusetts General Hospital CHGR NCL Disorders Clinical Database and Biorepository. HeLa cells (ATCC CCL-2) and human fibroblasts were grown and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES, and gentamicin at 37 °C in a humidified incubator with 5% CO_2. 50 μM CQ (MP Biomedicals) and 20 nM baf A (Cayman Chemicals), 50 μg/ml cycloheximide (Fisher Scientific), and 100 nM bortezomib (LC Laboratories) were used in the treatments. The CLN5 stable knockdown cell line was created by transfecting a plasmid containing shRNA sequence against CLN5 (SABiosciences) to HeLa cells and selecting for resistance to hygromycin. The SNCA knock-out HeLa cell line was generated using CRISPR/Cas9 method (plasmid was purchased from GeneCopoeia). The control siRNA, siRNA for SNCA (SMARTpool), and siRNA for CLN5 (GAACCUACUUAUCUGGGAA) were purchased from Dharmacon. RNAiMAX (Thermo Fisher) was used for siRNA transfections in HeLa and SH-SY5Y cells. The Neon electroporation transfection system was used for siRNA transfection in fibroblasts. For overexpression transfections, the TransIT-LT1 transfection reagent was used (Mirus Bio). The tandem mRFP-GFP-LC3 plasmid ptfLC3 was a gift from Tamotsu Yoshimori (Addgene plasmid #21074)[36]. The EGFP-Rab5 Q79L plasmid was a gift from Qing Zhong (Addgene plasmid #28046)[70].

Quantitative polymerase chain reaction (qPCR). RT² Profiler PCR Array of the human autophagy pathway was purchased from SABiosciences. RNA was isolated from fresh fibroblast pellets using RNeasy Mini Kit (Qiagen). 0.5 μg of RNA was used for the first strand cDNA synthesis (RT² First Strand Kit, SABiosciences). The cDNAs synthesized were used for RT² Profiler PCR Array with RT² SYBR Green qPCR Master Mixes samples. Three independent RNA prep samples were used for triplicate qPCR. Data was analyzed using RT² Profiler PCR Array data analysis tool. Fold-Change ($2^{(-\Delta\Delta Ct)}$) is the normalized gene expression ($2^{(-\Delta Ct)}$) in the CLN5-deficient fibroblast sample divided by the normalized gene expression ($2^{(-\Delta Ct)}$) of the control sample. The p values were calculated based on a Student's t-test of the replicate $2^{(-\Delta Ct)}$ values for each gene in the control group and the CLN5 patient group.

Sample preparation and Immunoblotting. Cells grown on culture dishes were scraped and washed once with 1X phosphate buffered saline (PBS), and centrifuged for 3 min at 1,500 × g. Cell pellets were lysed using RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (G-Biosciences). After incubation for 30 min on ice, extracts were centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant was collected as the whole cell lysates. Protein concentrations were determined by Bradford assay. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore) followed by immunoblotting. For the membranes that were prepared to blot for α-syn, after transfer, the membranes were first fixed with 1% formaldehyde in PBS for 30 min followed by blocking in 5% non-fat milk in TBST. ECL detection was performed according to the manufacturer's instructions (Millipore) and blots were imaged using G-Box (Syngene). Quantification was performed using ImageJ.

Immunofluorescence Microscopy. Cells on coverslips were fixed with 4% formaldehyde for 10 min at room temperature or 100% methanol for 15 min at −20 °C. Blocking, permeabilization, antibody incubations, and washes were done using blocking solution (10% fetal calf serum, 0.1% saponin, and 0.02% sodium azide in PBS). The cells were imaged using either Zeiss LSM-5 PASCAL or LSM-700 laser scanning confocal microscope. Quantification was performed using ImageJ. For comparison between groups, data was analyzed using a Student's t test with two-samples assuming equal variances. For comparison among multiple groups, a one-way ANOVA followed by the Tukey's post hoc test was performed.

Subcellular fractionation. After treatments, cell pellets were collected, resuspended in isotonic buffer (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, protease inhibitor cocktail), and incubated 10 min on ice. Samples were passed through a 25 G needle with 14 strokes. The post nuclear supernatant (PNS) was collected after centrifugation at 600 × g for 10 min at 4 °C. The PNS was centrifuged at 20,000 × g for 20 min at 4 °C. The supernatant was collected as the "soluble" fraction. The pellet was rinsed once with isotonic buffer and centrifuged again at 20,000 × g for 5 min at 4 °C. The pellet was resuspended in RIPA buffer as the "pellet" fraction. This is the crude membrane fraction that contains organelles (except nucleus and microsomes).

Antibodies. Rabbit polyclonal antibodies used in this study were against LC3B (Abcam, ab51520), and GRASP65 (Pierce, PA3-910). Rabbit monoclonal antibodies used in this study were against CLN5 (Abcam, ab170899) and α-synuclein (Abcam, MJFR1). Mouse monoclonal antibodies used in this study were against β-actin (GenScript, A00702), GAPDH (Developmental Studies Hybridoma Bank, DSHB-hGAPDH-4B7, deposited by DSHB), α-synuclein (Abcam, 4D6), P62 (Abcam, ab56416), GBA (Novus, MAB7410), and Lamp1 (DSHB, G1/139/5, deposited by Hauri, H.-P.)[71]. HRP-conjugated secondary antibodies for Western blotting were purchased from Jackson Laboratory. Secondary antibodies conjugated to Alexa Fluor 488 or 546 were purchased from Molecular Probes.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

J.A., M.F., T.B. and S.Y.L. conceived the project; J.A., M.F., J.A.M., A.R.W., B.A., T.B. and S.Y.L. performed the experiments; J.A., M.F., J.A.M., A.R.W. and S.Y.L. analyzed the data and wrote the paper.

Additional Information

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