HUMAN CYTOMEGALOVIRUS INDUCED CHANGES IN LOW-DENSITY LIPOPROTEIN RECEPTOR EXPRESSION AND TRAFFICKING

By

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Abstract

Human cytomegalovirus (HCMV) is a beta-herpesvirus that establishes a lifelong latent infection in its host. It then reactivates based upon environmental cues and stressors, causing disease in those who are immunocompromised. It has a broad cell tropism allowing it to infect a wide variety of cells and manipulate numerous cellular pathways to establish a successful infection. In unpublished data collected by the Goodrum lab, low-density lipoprotein receptor (LDLR) was found to be strongly downregulated in HCMV infection in fibroblasts. LDLR is a cell surface receptor responsible for the uptake of cholesterol-rich ligands. Using immunoblotting, immunofluorescence, and siRNA knockdowns, we gain insight into how LDLR is trafficked in a latent versus replicative infection, and why those changes may affect HCMV replication. In a latent infection, there is no change in LDLR levels between mock and WT infected cells. However, in a replicative infection in fibroblasts, mature LDLR (mLDLR) is driven to the lysosome for degradation, and immature iLDLR (iLDLR) accumulates in the ER and is targeted for ER-associated protein degradation (ERAD). A knockdown of LDLR increases viral protein levels and the cleavage of SREBP2, a transcription factor for lipogenic proteins. This may indicate that downregulating LDLR helps the virus increase cholesterol biosynthesis, providing a potential reason for the virus to degrade and block the formation of LDLR. Future experiments are needed to further understand the workings of the mechanism, and the implications and consequences of HCMV manipulating ERAD to degrade host proteins.

Introduction

Human cytomegalovirus (HCMV) is a beta-herpesvirus that infects 40-99% of the population, and establishes a lifelong infection in the host. It is also one of the largest viruses to infect humans, with a 236-kilobase genome that contains about 200 open reading frames (1). While most people who have HCMV are asymptomatic, newborns, those who are immunocompromised, and organ transplant recipients are most susceptible to complications caused by the virus. According to the CDC, approximately 1 in 200 newborns has a congenital CMV infection, and of those infected, 1 in 5 will develop long lasting health complications, such as hearing loss or cognitive impairment (2). This puts HCMV as the leading infectious cause of congenital neurological handicaps and disability. Similarly, HCMV is one of the leading infectious causes of negative outcomes related to solid organ and bone marrow transplants without antiviral prophylaxis (3). Therefore, there is a great need for the development of novel treatments, requiring the understanding of how HCMV can manipulate a host cell.

As with all herpesviruses, HCMV establishes a lifelong latent infection in the host where its viral genome is silently maintained without progeny production. Reactivation events of the virus can lead to the creation of new virus, potentially causing disease. This leads to HCMV having different infection kinetics and phenotypes reliant on whether the virus is in a latent or replicative state, which is dependent on cell type. Cells in the early myeloid lineage are primary targets for HCMV infection because they become latent reservoirs for the virus. HCMV latently infects CD34+ hematopoietic progenitor cells (HPCs) and its derivatives, such as CD14+ monocytes. Then, following a trigger such as monocyte differentiation, HCMV reactivates to a typical lytic infection, allowing for new virus production and infection of other organs (4, 5).

Many cell types also establish a replicative infection with viral transcription without first establishing a latent infection, and the rate of virions produced can vary. Fibroblasts support a highly replicative infection, producing abundant viral progeny, while endothelial cells support a slower, chronic infection (6).

In order to successfully survive in a host within multiple cell types with different infection dynamics, HCMV manipulates a wide variety of cellular systems in order to establish an infection. One major target of HCMV is cell surface proteins that are integral for antigen presentation, cell differentiation, metabolism, and more (7, 8). For example, HCMV-encoded proteins US2 and US11, drive the major histocompatibility complex (MHC) class 1 to the proteasome for degradation, thereby preventing antigen presentation and T-cell activity (9, 10). Another cell surface receptor called epidermal growth factor receptor (EGFR) regulates cell proliferation and differentiation, and it is hijacked by two viral proteins, UL138 and UL135. UL138 promotes latency, while UL135 drives reactivation from latency (11). EGFR at the cell surface is sustained by UL138, while UL135 interacts with the host proteins Abi-1 and CIN85 to internalize and turn over EGFR, providing one mechanism as to how the virus can control the shift from latency to reactivation (12, 13).

HCMV also governs processes responsible for de novo cholesterol biosynthesis and cholesterol uptake from external sources. Uptake, production, and regulation of cholesterol is essential for normal cell survival, as cholesterol has important roles in structuring cell membranes and producing hormones, vitamins, and other vital building blocks. It is well characterized that HCMV induces lipogenesis, the production of fats from fatty acids and glycerol, in order to aid in the production and release of viral progeny (14). HCMV partially

induces lipogenesis by cleaving sterol regulatory element binding protein 1 (SREBP1), a transcription factor that targets proteins involved in lipogenesis (15). One target protein is low-density lipoprotein receptor (LDLR), which is responsible for cholesterol uptake (16, 17). HCMV also uses inducible degrader of LDLR (IDOL) in a non-canonical role to degrade the viral protein UL136p33, promoting latency in cells (18). As IDOL is upstream of LDLR, and LDLR itself is a cell surface receptor important for host lipid homeostasis, the Goodrum lab decided to more thoroughly investigate HCMV modulation of LDLR.

LDLR is a cell surface receptor responsible for the clathrin-mediated endocytosis of cholesterol-rich ligands, such as low-density lipoprotein (LDL), alongside other receptors with similar functions such as very low density lipoprotein receptor (VLDLR) and Apolipoprotein E receptor 2 (ApoER2) (19, 20). The extracellular N-terminal of LDLR is called the ligand binding domain (LBD) and contains disulfide bonds and calcium ions needed for structural integrity and binding ligands. Then, there is a epidermal growth factor precursor homology domain, a O-linked sugar region with an unknown function, a transmembrane domain to anchor the protein in a lipid bilayer, and a C-terminal cytosolic domain important for endocytosis (21). LDLR is synthesized in the endoplasmic reticulum (ER) and is co-translationally N-linked glycosylated. Then, it is trafficked to the Golgi, where O-linked glycosylation happens post-translationally (22). This completes the LDLR maturation process, where Rab3b transports LDLR directly from the trans-Golgi network (TGN) to the plasma membrane (PM) or Rab13 indirectly transports LDLR to the PM through trafficking to a recycling endosome, which will take LDLR to the PM (23).

Once on the surface of the cell, it is able to bind to a LDL molecule, causing the endocytic adapter proteins autosomal recessive hypercholesterolemia (ARH) or Disabled-2 (Dab2) to bind to a NPXY motif in the C-terminal of LDLR (24, 25). This triggers clathrin-dependent endocytosis of the LDLR complex, where the clathrin-coated vesicles fuse with early endosomes. The acidic environment of the early endosome causes separation of the ligand and receptor, and while LDL is eventually degraded in the lysosome, LDLR is trafficked to a recycling endosome and returns to the cell surface (26).

In addition to recycling, LDLR can also be internalized for degradation. It has two degraders, Inducible Degrader of LDLR (IDOL) and Proprotein convertase subtilisin/kexin type 9 (PCSK9). IDOL is an E3 ubiquitin ligase that ubiquitinates cell surface LDLR for degradation through the lysosome (27). PCSK9 can target LDLR both in the TGN intracellularly and on the cell surface extracellularly by binding to the EGF-like domain, with both pathways bringing LDLR to the lysosome for degradation (28, 29, 30). To prevent immediate breakdown of LDLR, LDL can bind to PCSK9 to prevent LDLR degradation (31).

In preliminary studies done by the Goodrum lab, the mature, fully glycosylated form of LDLR (mLDLR) is downregulated in fibroblast infection, and this loss is independent of PCSK9 and IDOL. However, later during the infection cycle, there is a simultaneous accumulation of the immature form of LDLR (iLDLR), suggesting there is a block in the maturation process of LDLR. Overall, the purpose of this project is to help determine how HCMV is trafficking LDLR across cell types, and to create a LDLR KO to determine if the downregulation of LDLR is pro or antiviral.

Materials and Methods

Cells and Virus. MRC-5s, human fetal lung fibroblast cells, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-alanyl-glutamine, 100 Units/ml penicillin, and 100 µg/ml streptomycin. THP-1 monocytes were cultured in RPMI with 10% fetal bovine serum, 1mM sodium pyruvate, 100 Units/ml penicillin, 100µg/mL streptomycin, and 50µM BME. At 5dpi, infected monocytes are treated with 100nM of 12-O-Tetradecanoylphorbol-13-acetate (TPA) or a DMSO solvent control to promote monocyte to macrophage differentiation. Endothelial cells, Human umbilical vein endothelial cells (HUVECS), were cultures in supplemented EMB-2™ Basal Medium (Lonza). BAC clones of the HCMV TB40/E WT virus were a gift from Christian Sinzger at Institut für Medizinische Virologie in Germany, and were engineered to express green fluorescent protein (GFP) (32). Infection was monitored through GFP expression. All infections were done at a multiplicity of infection (MOI) of 1 unless otherwise stated.

Immunoblotting. 50 µg of protein lysate in 4X Sodium dodecyl sulfate were loaded on a 12% Tris-Bis gel and separated by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane activated with methanol, and were blocked with 5% BSA in TBS-T for 1 hour at room temperature. Protein-specific primary antibody was added for either 1 hour at room temperature or overnight at 4°C. Fluorescent secondary antibody was then added for 1 hour at room temperature, and then imaged using an Odyssey infrared imaging system (LI-COR Biosciences). All antibodies and sources are listed in Table 1.

Table 1. Antibody description and sources

Antigen	Antibody	Concentration	Animal	Source
LDLR (Immunoblotting)		1:1,000	Rabbit	Proteintech
α -Tubulin	DM1A	1:2,000	Mouse	Cell Signaling Technology
IE1/IE2	3H4	1:100	Mouse	Gift, Dr. Thomas Shenk through John Purdy
pp150		1:50	Mouse	Gift from Dr. William Britt
pUL135		2 µg/ml	Rabbit	Open Biosystems
pp28 (UL99)	10B4-29	1:50	Mouse	Gift, Dr. Thomas Shenk
ApoER2	EPR3326	1:1,000	Rabbit	Abcam
SREBP2		1:1,000	Rabbit	Abcam
DyLight™ 800 anti rabbit (Immunoblotting)		1:5,000	Goat	Invitrogen
DyLight™ 680 anti mouse (Immunoblotting)		1:6,000	Goat	Invitrogen
LDLR with conjugated APC (IF)		1:200	Rabbit	Sino Biological
GM130	Clone 35	1:100	Mouse	BD Biosciences
Calnexin	C5C9	1:100	Rabbit	Cell Signaling Technology
AlexaFluor™ 546 Anti rabbit (IF)		1:1,000	Goat	Invitrogen
AlexaFluor™ 546 Anti mouse (IF)		1:1,000	Goat	Invitrogen

Immunofluorescence. MRC-5s were plated on cover slides and infected the next day at a MOI of 2. At 24, 48, or 72 hours post infection (hpi), the cells were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde at room temperature for 10 minutes. The cells

were permeabilized with 0.1% Triton X-100 in PBS for 10 min, placed in 50mM glycine for 5 minutes, washed with PBS, and then placed in blocking buffer for 1 hour at room temperature. Primary antibody was then added for one hour at room temperature, before being wash 3 times for 5 minutes with PBS. Secondary antibody was added for 1 hour at room temperature. In the first wash of PBS, 1 ng/µl of 4',6'-diamidino-2- phenylindole (DAPI) is added, followed by two more washes of PBS. Cover slides were then placed in the dark to dry, before being mounted with ProLong™ Gold antifade mountant. All images were taken on Deltavision deconvolution microscope.

CRISPR KO Cloning and Transformation. lentiCRISPRv2 plasmid was dephosphorylated and digested and with enzymes AP and BsmBI and run on a .7% agarose gel. The plasmid was purified using the Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel). 2 different sets of LDLR gRNA and one set of Luciferase gRNA for the CRISPR KO targeting different sites were designed, and the forward and reverse oligo for each KO were phosphorylated using the enzyme PNK and annealed together. Then, the digested lentiCRISPRv2 plasmid and oligo duplexes were ligated together with a ligase enzyme. This final plasmid was transformed into chemically competent BL21(DE3) Escherichia coli before being plated on Lysogeny broth (LB) agar plate supplemented with 10% ampicillin.

Lentivirus Transfection. 8µg of LDLR and Luciferase CRISPR KO plasmids were each combined with 5.18µg of psPAX2 plasmid (Addgene) and 2.81µg of pMD2.G plasmid (Addgene) in 122µL of Opti-MEM™ (Thermo Fischer Scientific) media, before being mixed with 53.28µl of

polyethyleneimine (PEI). The mixture was vortexed, and after waiting 10-20 minutes, added to HEK293T cells on 10cm dishes at 70-75% confluency. The next day, the media on the cells was changed to 2x HEPES-supplemented media. The media was collected 48 and 72 hours after transfection and stored at 4°C until it was spun down in an ultracentrifuge at 17,000xg at 4°C for 2 hours. The supernatant was aspirated and the pellet was resuspended in DMEM and placed on a shaker for 1 hour at 4°C. 50µL aliquots of the lentivirus were then stored in a -80°C freezer until ready to be transduced.

Lentivirus Transduction. Lentivirus was thawed from the -80°C, thawed on ice, vortexed briefly, and then spun in a centrifuge for 1 minute at 10,000xg to get rid of any leftover cell debris. The lentivirus was then added to DMEM media with 8µg/ml of polybrene and placed on MRC-5s. Two days later, 2µg/mL of puromycin to select cells with successful incorporation of the knockout.

LDLR knockdown. Reverse transfection of LDLR and nontargeting control (NTC) siRNAs were performed using Lipofectamine RNAiMAX reagent. Mix 1 was prepared by adding 5µl RNAiMAX to 125µl Opti-MEM medium. Mix 2 was prepared by adding 30pmol of siRNA was added to 125µl Opti-MEM medium, and Mix 2 was added to Mix 1. This was added to a well in a 6-well dish, and incubated for 10 minutes at room temperature. Approximately 300,000 fibroblasts in DMEM media were added to each well and incubated at 37°C overnight. The next day, new DMEM was added to the cells, and 48 hours after the reverse transfection, fibroblasts were infected as stated above.

Results

HCMV induced LDLR phenotypes are determined by infection state. Early experiments indicate there are lower levels of the mature form of LDLR in HCMV infection not reliant on LDLR's normal degraders, IDOL or PCSK9. It has been previously shown that HCMV has a broad cell tropism, so we examined LDLR expression across cell types that support a highly replicative, chronic, or latent wildtype (WT) infection (33). In fibroblasts that support a highly replicative infection, there is a steady decrease of mLDLR expression over 24-72 hours post infection (hpi) in comparison to mock fibroblasts (Fig. 1A). At 48hpi, there is the appearance of an immature band of LDLR not present in mock-infected fibroblasts, which is sustained to 72hpi. Infected endothelial cells that support a slower replicative infection show a similar pattern to fibroblasts. While the downregulation of mLDLR is not as profound in endothelial cells compared to fibroblasts, there is still a loss of mLDLR in infection (Fig. 2B). iLDLR accumulation in endothelial cells begins earlier at 24hpi, and continues to 48hpi.

While fibroblasts and endothelial cells model productive infections, THP1 monocytes are used to model HCMV latency and subsequent reactivation (34). After establishing a latent infection for 5 days, TPA is added to half the THP1s to promote monocyte-to-macrophage differentiation and stimulate viral reactivation. The remaining THP1s are treated with DMSO as a solvent control and continue maintaining a latent infection. At 1 day post infection (dpi), mLDLR is increased in WT relative to mock infected THP-1s, and for the ensuing latent infection, levels of mLDLR are similar between WT and mock (Fig. 1C). There is also no additional buildup of iLDLR in a latent WT infection. When examining all differentiated THP1's at 7 and 8dpi, there is a considerable decrease of LDLR independent of infection, likely due to the drastic changes in

gene expression patterns that typically occur in monocyte-to-macrophage differentiation (35). Comparing WT and mock differentiated monocytes reveals they have similar levels of mLDLR. However, there is an increase of iLDLR at 8dpi in the reactivated WT infected monocytes compared to the mock monocytes, which is similar to the pattern shown in fibroblasts and endothelial cells. Collectively this data suggests that the decrease in mLDLR expression and increase in iLDLR expression only occurs in actively replicative infections.

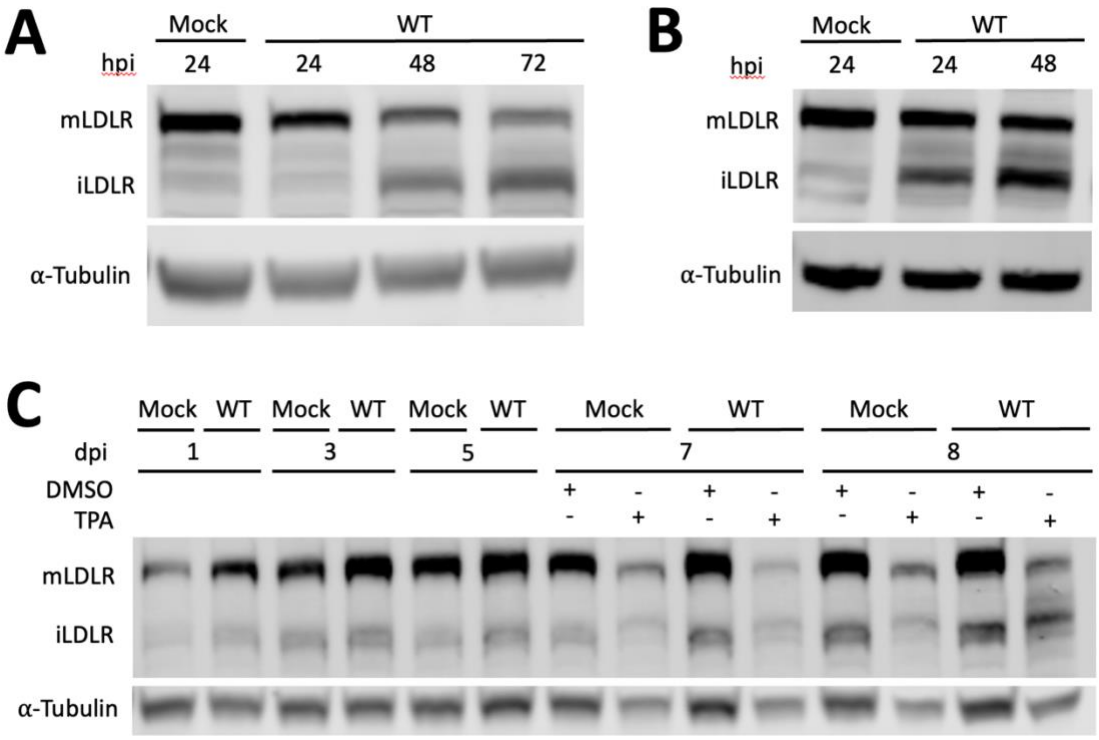

Figure 1. HCMV induces changes in LDLR expression dependent on infection state. (A) Fibroblasts were mock-infected or infected with WT virus and protein lysates were harvested at 24-72 hpi. (B) Endothelial HUVEC cells were mock-infected or infected with WT virus and protein lysates were harvested at 24-48 hpi. (C) THP-1 monocytes were mock-infected or infected with WT virus for 1-8 dpi and protein lysates were harvested at 1,3,5,7, and 8dpi. After establishing a latent infection, at 5dpi THP-1s were either treated with 100 nM TPA to promote monocyte differentiation and stimulate HCMV reactivation or DMSO as a solvent control. At each time point for all samples, cells were lysed and immunoblotted for LDLR as well as α-Tubulin, a loading control.

There are no changes in ApoER2 levels during HCMV infection. Since LDLR levels are being dowregulated in a replicative infection, we examined another lipoprotein receptor, ApoER2, to see if this response is specific to LDLR, or if other proteins with a similar function have a change of expression in infection. ApoER2 levels across a timecourse of WT infected fibroblasts remain consistent with mock infected levels, implying the downregulation of LDLR is not just a broader response to reduce all cholesterol cell surface receptors (Fig. 2).

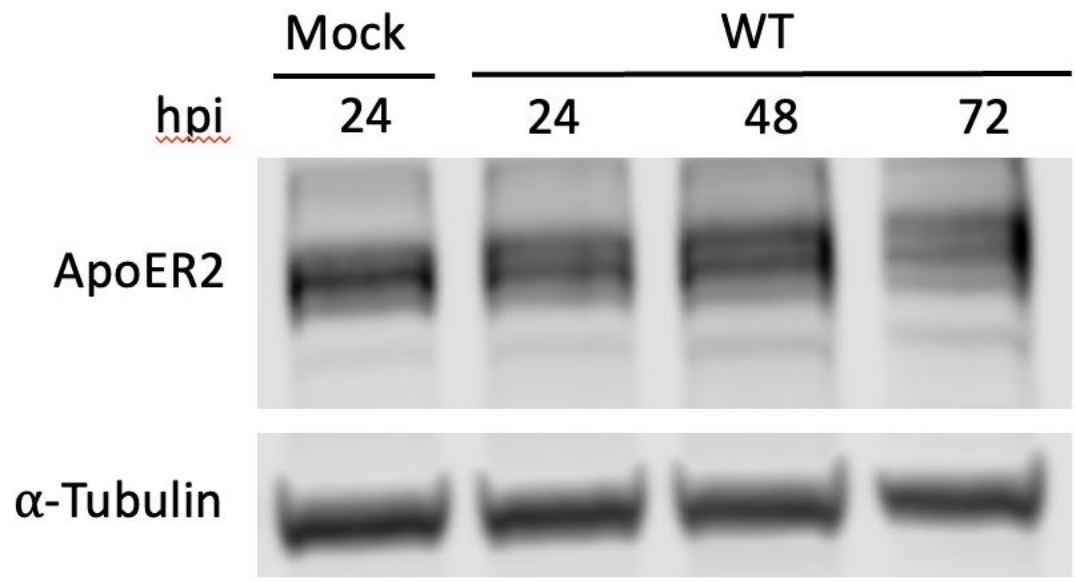

Figure 2. A replicative HCMV infection does not change ApoER2 expression. Fibroblasts were mock-infected or infected with WT virus and protein lysates were harvested at 24-72 hpi. At each time point for all samples, cells were lysed and immunoblotted for ApoER2 as well as α-Tubulin, a loading control.

HCMV infection targets mLDLR for degradation via the lysosome. Upon observing that mLDLR protein levels decrease in a replicative WT infection, we wanted to determine how HCMV downregulates mLDLR levels. Since LDLR is degraded via the lysosome under normal

cellular conditions, to determine if HCMV hijacks this pathway to reduce mLDLR expression, we infected fibroblasts and inhibited the lysosome with 0.2µM Bafilomycin (BafA) and looked for rescue of mLDLR levels. In mock infected cells, there was the expected rescue of mLDLR with the addition of BafA (Fig. 3). Rescue of mLDLR was also seen at all timepoints across infection from 24-72hpi, suggesting HCMV drives downregulation of mLDLR by trafficking it to the lysosome for degradation.

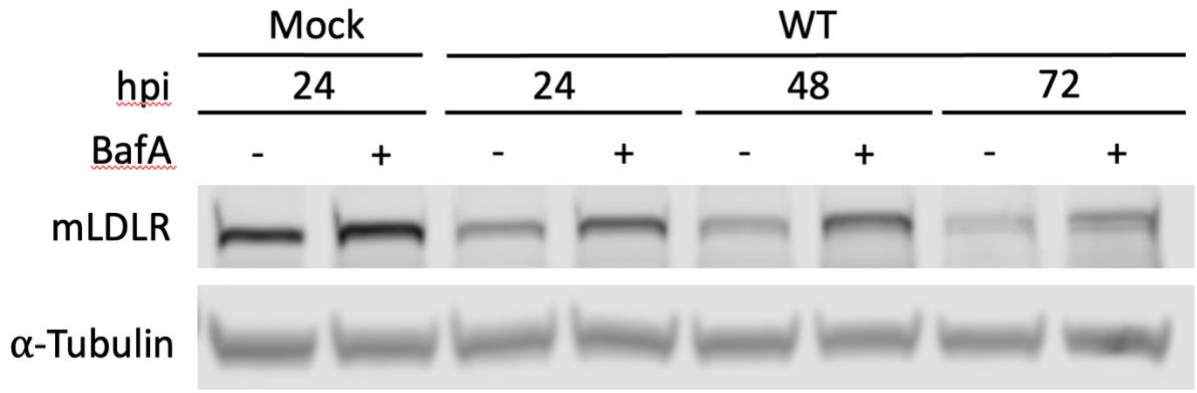

Figure 3. HCMV degrades mLDLR via the lysosome. Fibroblasts were mock-infected or infected with WT virus for 24-72hpi. Cells were treated with 0.2µM Bafilomycin (BafA) 6 hours prior to lysis of protein lysate collection. Proteins were detected by immunoblotting for LDLR as well as α-Tubulin.

mLDLR downregulation in infection is not dependent on late viral gene expression. Since we determined that HCMV degrades mLDLR via the lysosome throughout a 72 hour time course, next we wanted to investigate what stage of infection is primarily responsible for producing this phenotype. Downregulation of LDLR continues strongly through later time points in infection, such as 72hpi, when late viral proteins are expressed. To determine if late viral gene expression is necessary for the continued downregulation of LDLR, we added an inhibitor

of late viral gene expression, phosphonoacetic acid (PAA), and examined whether there was less downregulation of mLDLR.

Infected cells exhibit the same phenotype as infected cells treated with PAA, with a sustained downregulation of LDLR through 72hpi (Fig. 4). However, in PAA treated cells, iLDLR levels decrease, potentially indicating that late viral proteins are not needed for targeting mLDLR for degradation, but that they assist in accumulation of iLDLR.

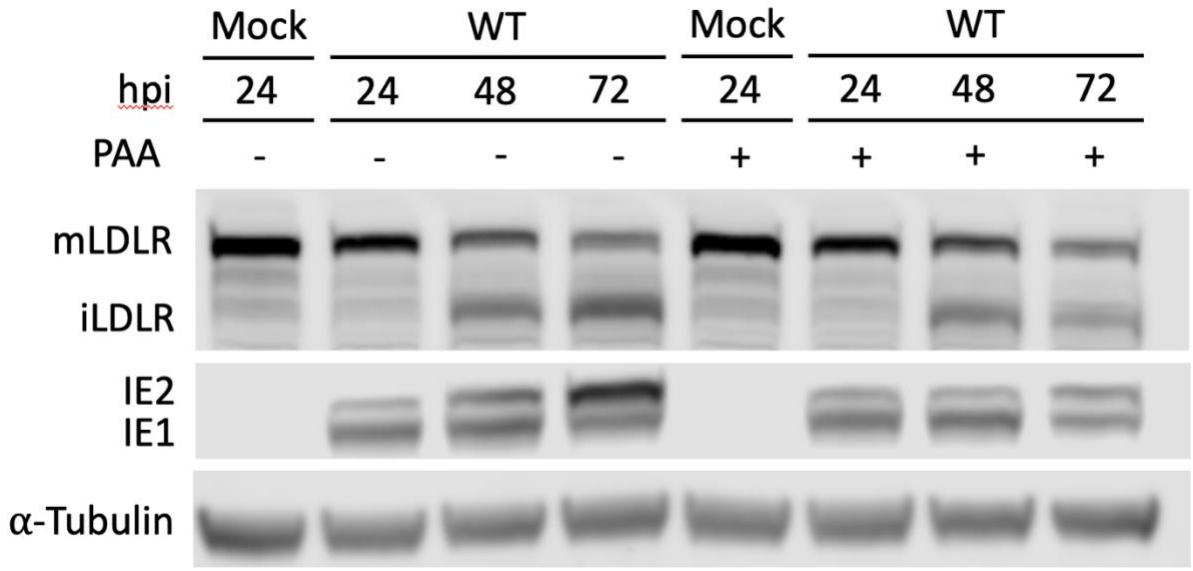

Figure 4. Late viral gene expression is not necessary for the downregulation of mLDLR. Fibroblasts were mock-infected or infected with WT virus, and 2 hours later, 100μM phosphonoacetic acid (PAA) was added as shown. Protein lysates were harvested at 24-72 hpi, and proteins were detected by immunoblotting for LDLR, α-Tubulin, as well as IEs as a control for PAA treatment.

LDLR CRISPR KO was unable to fully knockout LDLR protein expression in fibroblasts. The observation that iLDLR accumulation partially depends on a late viral protein suggests that the virus is somehow preventing maturation of LDLR. Therefore, we wanted to investigate if the

downregulation of mLDLR is pro-viral as well, or if downregulation is a cellular response for infection.

To achieve this, we created a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated protein (Cas9) knockout (KO) for LDLR as well as a Luciferase control. The CRISPR-Cas9 system is used to edit genomes. A guide RNA (gRNA) directs Cas9 to the gene encoding a protein of interest, where Cas9 cuts that section of DNA. The cell repairs the break, but often times this causes an insertion or deletion of nucleotides, leading to an unfunctional protein, effectively getting rid of the cell's ability to create the protein of interest (36). A Luciferase control encodes for guide RNA (gRNA) that should not change the protein of interest's levels, so it is used to account for any off-target effects due to the processes of transducing the cells with the CRISPR KO. Using a CRISPR KO to eliminate LDLR expression in fibroblasts, we aimed to measure the output of viral titers in Luciferase KO vs LDLR KO fibroblasts in order to determine if LDLR is helpful or inhibitory to viral replication.

Fibroblasts were transduced with either the lentivirus containing the Luciferase KO or LDLR KO at a MOI of 6, and successfully transduced cells were selected for using puromycin (Fig. 5A). However, upon confirming the knockout on a Western blot, it was revealed that there was only a 38% decrease in LDLR protein expression in the LDLR KO versus the Luciferase KO, suggesting that only one allele of LDLR was targeted (Fig. 5B). Since there was insufficient knockout of LDLR, we looked at other experimental ways we could determine if the downregulation of LDLR in infection is pro or antiviral.

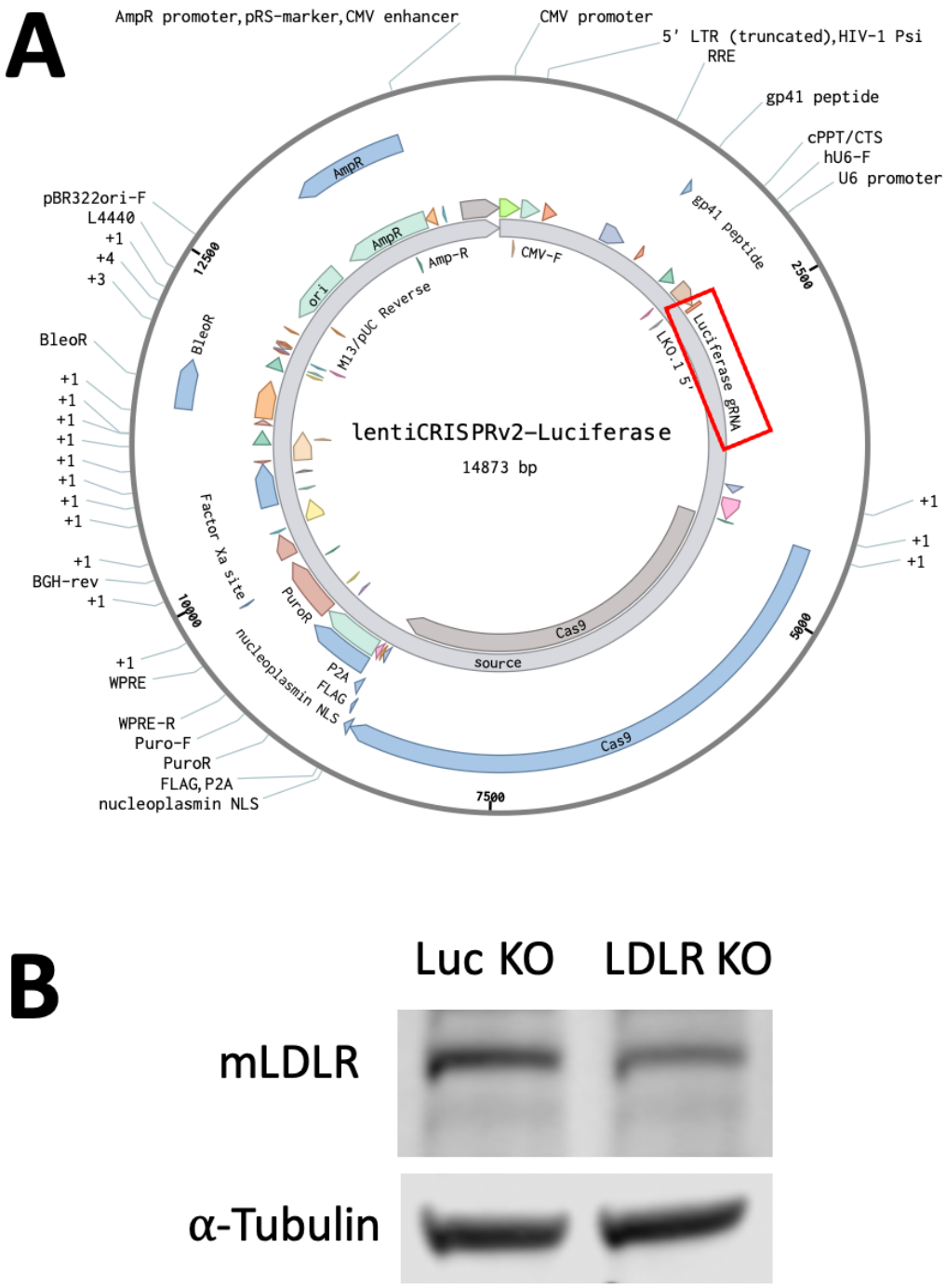

Figure 5. Creation and Test of CRISPR LDLR KO. (A) Vector map of lentiCRISPRv2-Luciferase plasmid. The placement of the Luciferase, or the LDLR, gRNA sequence is in the red box and the Ampicillin resistance gene (AmpR) is shown in blue alongside the gene sequence for Cas9. (B) Fibroblasts were transduce with either the Luciferase KO or LDLR KO lentivirus at a MOI of 6, and 2 days later were treated with 2µg/mL of puromycin to select for cells that had successfully incorporated the KO into their genome. Knock out was confirmed by immunoblotting for LDLR.

Lower mLDLR protein levels increase late viral protein expression and SREBP2 cleavage. Since a complete KO of LDLR could not be achieved, a small interfering RNA (siRNA) knockdown (KD) of LDLR was used to reduce LDLR expression instead. Adding siRNA activates the RNA-induced silencing complex (RISC) to degrade complementary mRNA to the siRNA, reducing the levels of the corresponding protein made from that mRNA (37). We created siRNA to target mRNA encoding for LDLR, in addition to a nontargeting control (NTC) siRNA that does not target LDLR, therefore accounting for any off-target effects of adding siRNA to fibroblasts.

To determine whether the degradation of mLDLR in a replicative infection is pro or antiviral, we compared a variety of early and late viral protein levels between the LDLR KD and NTC KD at an early timepoint, 24hpi, and late timepoint, 96hpi. The first stage of HCMV infection is the expression of immediate early genes and proteins, including immediate early proteins 1 (IE1) and 2 (IE2) (38). Then, reliant on immediate early genes, early genes are expressed. UL135 is an early protein that assists in establishing a replicative infection (11, 39). Pp150 and pp28 are tegument proteins that accumulate late during viral infection, where they are primarily involved in the assembly and egress of virus particles (40).

Immediate early proteins IE1 and IE2, as well as the early protein UL135, have the same amount of expression in the NTC vs LDLR KD WT infected cells across all timepoints (Fig. 6A). However, the late proteins pp150 and pp28 are at higher levels in the LDLR KD relative to the NTC KD at 96hpi, suggesting that lower levels of LDLR may allow for a more replicative viral program, which drives increases in late viral protein accumulation.

Since LDLR is a cholesterol receptor, we also examined SREBP2 levels in NTC vs. LDLR KD fibroblasts. SREBP2 is a transcription factor primarily involved in regulating cholesterol

synthesis (41). We found that there are consistently higher levels of cleaved SREBP2 in LDLR KD vs. NTC fibroblasts (Fig. 6B).

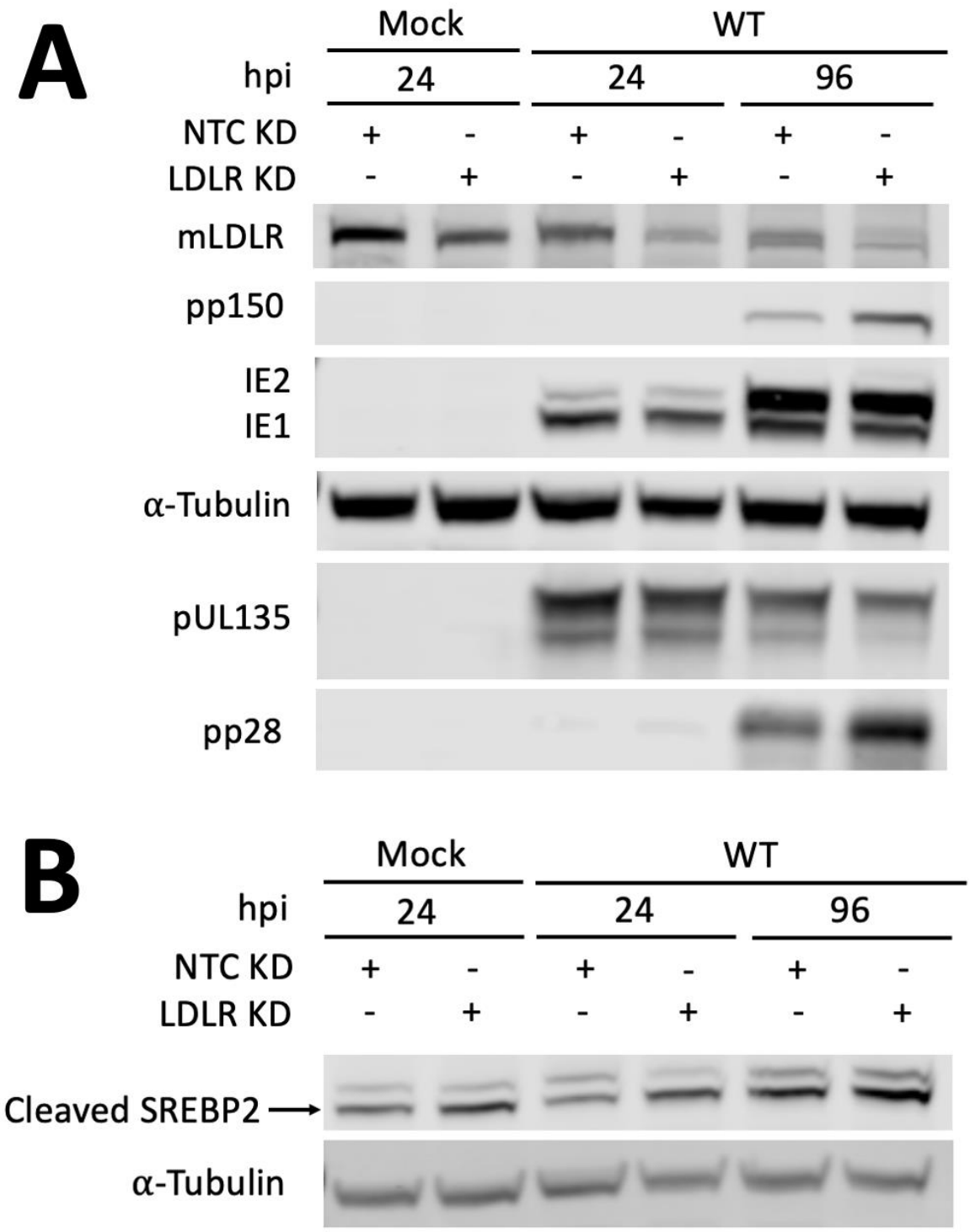

Figure 6. LDLR KD increases late viral protein expression and SREBP2 cleavage. To analyze the effects of a LDLR knockdown, fibroblasts were reverse transfected with LDLR or nontargeting control (NTC) siRNA as shown. Two days later, they were infected with WT virus for 24-96hpi, and each time point for all samples, cells were lysed and immunoblotted for (A) LDLR, pp150, IE1&2, pUL135, pp28 and α-Tubulin or (B) cleaved SREBP2 and α-Tubulin.

At 48hpi LDLR is colocalized with the ER. So far we have determined that HCMV targets mLDLR for degradation by the lysosome, and that this is a pro-viral phenotype resulting in higher levels of late viral proteins. In order to gain a broader perspective on the role of LDLR in a replicative infection, we started examining the iLDLR that accumulates at 48hpi in Western blots. The iLDLR band that appears in infected fibroblasts runs at about 110 kDA while the mLDLR band runs around 150 kDA. We postulate that the 110kDA band is the unglycosylated form of LDLR and that there could be an accumulation of LDLR in the ER or Golgi, where HCMV is blocking normal glycosylation and preventing the maturation of LDLR. To test this, we visualized LDLR with ER and Golgi apparatus markers using IF to see if there was any difference in colocalization between mock and WT infected fibroblasts.

Since the accumulation of iLDLR is prominent at 48hpi, we picked this time point to perform immunofluorescence (IF). In fibroblasts labeled with antibodies for LDLR and a Golgi apparatus marker, GM130, there is very little colocalization in either the mock or WT infected fibroblasts (Fig. 6A). One visual hallmark of HCMV infection is the appearance of a kidney-shaped nucleus at 48hpi that forms around the viral assembly compartment (VAC), which is responsible for viral assembly and egress (42). Although LDLR is not colocalized with the Golgi apparatus at 48hpi, LDLR does appear to be in the area where the VAC is (Fig. 7A).

At 48hpi in mock fibroblasts, there is some colocalization of LDLR and the ER around the nucleus, where Calnexin is used as the ER marker. In WT-infected fibroblasts, there is more colocalization of LDLR and the ER both around the nucleus and farther out into the cytoplasm of the cell (Fig. 7B). Comparing between mock and WT infected cells at 48hpi, there is more colocalization between LDLR and ER in WT infected cells.

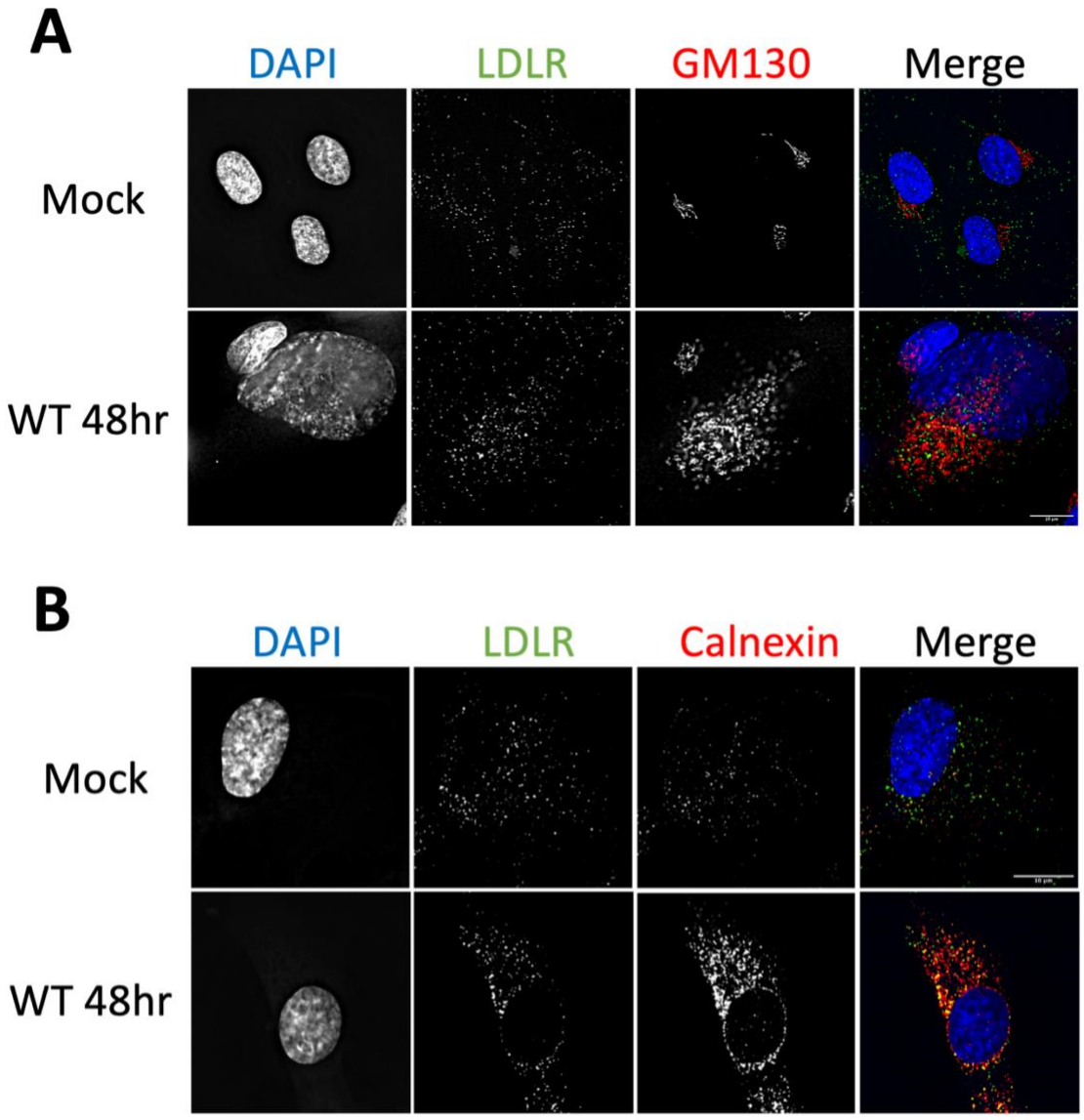

Figure 7. LDLR is colocalized with the ER at 48hpi. Fibroblasts were plated on chamber slides at 20,000 cells per well, and infected the next day at an MOI of 2. They were fixed with 4% PFA at 48 hours and LDLR was localized by immunofluorescence with a (A) Golgi apparatus marker, GM130, or (B) an ER marker, Calnexin. DAPI staining marks the nuclei.

Discussion

As with all herpesviruses, HCMV is able to manipulate complex cellular pathways in order to establish a lifelong infection in its host. It establishes latency in monocytes and CD34+ hematopoietic progenitor cells (HPCs), and then reactivates upon certain environmental cues or stressors to produce viral progeny and infect other types of tissue (4,5). To successfully establish latency, evade the immune system, and reactivate and replicate, HCMV controls many cell surface receptors, such as EGFR and MHC class 1 (9, 10, 12). Latently infected cells have sustained EGFR on the cell surface, but sequestering EGFR internally helps the virus transition to a replicative infection (13). HCMV also degrades host MHC class 1 while encoding viral MHC class 1 mimics to avoid detection from natural killer cells (43).

In this study, we examine how HCMV alters the trafficking and expression of LDLR, a cell surface protein that binds to cholesterol and brings it into the cell via endocytosis. Cholesterol metabolism is another essential cellular system that HCMV exploits, as cholesterol is an important element in producing viral progeny (14). Therefore, we wanted to explore the possibility that cholesterol metabolism is partially regulated by manipulation of lipoprotein receptors on the cell surface.

Since there are multi-faceted changes in viral and cellular protein expression during the transition from latency to reactivation, we first immunoblotted for LDLR in 3 different cell types that each support a different infection type. THP1 monocytes that support latency show no change in LDLR, while fibroblasts and endothelial cells that support an active infection show a downregulation of the fully mature form of LDLR, as well as the buildup of a smaller form of LDLR, which we believe is the unglycosylated, immature form of LDLR (Fig. 1). After reactivation

of HCMV in differentiated monocytes, there is a slight accumulation of iLDLR. These findings suggest that HCMV only influences LDLR expression in an active infection, supporting an idea that cholesterol metabolism is manipulated by HCMV to assist in the production of new virions, a process that only occurs in an active infection. However, ApoER2, another lipoprotein family receptor closely related to LDLR, was unaffected in infection (Fig. 2). This implies LDLR plays a particular role in infection that HCMV has specifically evolved to modulate.

Following this data, we began to explore how and why LDLR expression levels decline in active infection in fibroblasts. First, we observed that specific small molecule inhibition of lysosomal degradation showed increased rescue of mLDLR protein, leading us to conclude that mLDLR is degraded by the lysosome in infection (Fig. 3). Since there is a larger rescue of LDLR in the WT versus mock infected samples, it suggests there is not just a blockage of the maturation of LDLR, but a specific downregulation of the mature form as well. A viral mechanism may be responsible for redirecting LDLR either from recycling endosomes or the plasma membrane to the lysosome, accounting for the distinct expression changes in infection.

Next, we aimed to test whether this phenotype is driven by HCMV, or if it is cellular response to infection. At first, there was an attempt to determine what specific viral protein, or group of viral proteins causes the downregulation of mLDLR. We blocked late viral gene expression to see if that would have an effect on the lysosomal targeting of mLDLR, but no change was seen, meaning the downregulation of mLDLR is not reliant on late viral proteins (Fig. 4). If this is driven by HCMV, then this would suggest an early protein can drive downregulation of mLDLR via the lysosome. This would indicate that LDLR may play an antiviral role in HCMV replication, and therefore HCMV has evolved mechanisms to decrease LDLR

expression at the protein level. To test the idea of LDLR as an antiviral protein, we examined how removing LDLR before infection would change viral phenotypes. Although a successful CRISPR knockout wasn't achieved, a knockdown of LDLR via siRNA gave about 40% knockdown at the protein level, and in these cells we saw increased levels of late viral proteins (Fig. 5-6A). This, along with other data that shows a 10-fold increase in viral titers in the LDLR KD vs nontargeting control KD, argues that the decrease in mLDLR is driven directly by HCMV to benefit viral replication and potentially gene transcription in general. However, we have been unable to block HCMV driven downregulation of LDLR to further support that downregulation of LDLR is necessary for HCMV replication.

The decrease in viral titers and increase in viral protein expression in LDLR KD cells suggest that removal of mLDLR may stimulate viral transcription in some way. A potential mechanism for this arises when noticing that in a LDLR KD, there are higher levels of SREBP2 cleavage (Fig. 6B). In uninfected cells, SREBP2 is a transcription factor that resides in the ER until low levels of sterols are detected, in which case it is translocated to the Golgi and cleaved by site-1 protease (S1P) and site-2 protease (S2P) (44). Cleaved SREBP2 then enters the nucleus and promotes the transcription of proteins involved in de novo biosynthesis of lipids, such as cholesterol (45). As mentioned previously, cholesterol is vital for viral progeny production, and HCMV is already known to drive SREBP1 cleavage to promote lipogenesis (15). Therefore, it is possible by downregulating LDLR, a cell surface receptor for cholesterol, this promotes synthesis of cholesterol in the cell. A similar mechanism has been described in a related gammaherpesvirus murine gammaherpesvirus 68 (MHV68), where LDLR protein levels in primary macrophages are also downregulated by MHV68 to ultimately increase cholesterol

biosynthesis (46). Nevertheless, more experiments are needed to support this theory in HCMV. A cholesterol enzyme-linked immunosorbent assay (ELISA) comparing cholesterol levels between NTC KD versus LDLR KD fibroblasts would be able to determine if a reduction in mLDLR promotes more cholesterol in the cell. If so, we would propose this occurs through driving transcriptional responses that increase biosynthetic enzymes that would trigger increases in fatty acid and cholesterol that HCMV can utilize.

Another potential reason that mLDLR levels decrease early infection could be that LDLR is an entry receptor for HCMV viral particles. Hepatitis C virus and vesicular stomatitis virus, both single-stranded RNA viruses, use mLDLR as a cellular receptor for entry (47, 48). While this could explain the early downregulation of mLDLR, the mechanism for why mLDLR is still highly downregulated at 72 hours would still have to be determined.

In addition to mLDLR being degraded in the lysosome throughout infection, possibly by an early viral protein, there is also an accumulation of iLDLR in the ER (Fig. 7). Not only is HCMV downregulating any mLDLR on the cell surface, but it is also preventing mLDLR from being made. Additional findings showing iLDLR is degraded via the proteasome in a UL138-dependent manner rather than through the lysosome suggests iLDLR is being targeted for ER-associated protein degradation (ERAD). ERAD is a cellular pathway responsible for destroying and recycling misfolded proteins stuck in the ER (49). HCMV is already known to induce ERAD to degrade proteins related to the immune response, such as MHC class 1, and retroviruses have also been reported to degrade CD4 receptors in a similar process to ERAD (50). UL148, another HCMV viral protein, is known to interact with SEL1 to decrease ERAD degradation of the unstable viral glycoprotein gO needed for cellular entry (51). In mass spectrometry data collected by the

Goodrum Lab, UL138 interacts with UL148, SEL1, VCP, and GRP94, a variety of viral and host proteins known to regulate ERAD. Therefore, investigating how UL138 and LDLR are linked within an ERAD context could provide insight into how the synthesis of host proteins is manipulated in infection.

In conclusion, we determined that mature LDLR is targeted for rapid degradation by the lysosome in replicative HCMV infections. This response is specific to LDLR, and expression of similar receptors is not changed in infection. While the viral protein or proteins regulating LDLR have yet to be determined, it is likely they are early viral proteins. Removing LDLR increases the amount of viral proteins, and while one hypothesis is that it changes cholesterol metabolism to increase biosynthesis of cholesterol, further experiments are needed to determine if this is the case. Immature forms of LDLR also accumulate in the ER and are targeted for ERAD. These results show new insights into how HCMV is able to manipulate cellular trafficking pathways and lead to a deeper understanding of more effective ways to treat an active HCMV infection.

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