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Proteomic Identification of Nuclear Processes Manipulated by Cytomegalovirus Early during Infection

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Abstract

Human cytomegalovirus (HCMV) is a herpesvirus that is ubiquitously distributed world-wide and causes life-threatening disease upon immunosuppression. HCMV expresses numerous proteins that function to establish an intracellular environment that supports viral replication. Like most DNA viruses, HCMV manipulates processes within the nucleus. We have quantified changes in the host cell nuclear proteome at 24 hpi following infection with a clinical viral isolate. We have combined SILAC with multiple stages of fractionation to define changes. Tryptic peptides were analyzed by RP-HPLC combined with LC-MS/MS on an LTQ Orbitrap Velos mass spectrometer. Data from three biological replicates were processed with MaxQuant. A total of 1281 cellular proteins were quantified and 77 were found to be significantly differentially expressed. In addition, we observed 36 viral proteins associated with the nucleus. Diverse biological processes were significantly altered including increased aspects of cell cycling, mRNA metabolism, and nucleocytoplasmic transport while decreased immune responses. We validated changes for several proteins including a subset of classical nuclear transport proteins. In addition, we demonstrated that disruption of these import factors is inhibitory to HCMV replication. Overall, we have identified HCMV-induced changes in the nuclear proteome and uncovered several processes that are important for infection.

Keywords

nucleus; proteome; cytomegalovirus; import; infection

1 Introduction

Human cytomegalovirus (HCMV) is a β -herpesvirus that infects the majority of the world population (reviewed in [1]). The virus causes life-threatening disease in immunocompromised individuals and is a leading cause of congenital birth defects. Similar to all herpesviruses, primary exposure to HCMV results in a life-long infection consisting of lytic and latent replication states. Chronic infection has been associated with diverse pathologies. Antiviral compounds have been developed that significantly improve the

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management of HCMV disease. However, several limitations exist including toxic side effects, poor oral bioavailability, and the emergence of drug resistant strains. More effective strategies to manage infection will be possible by defining the complex relationship between the virus and host cellular processes.

HCMV requires the host cell nucleus for multiple stages of the replication cycle. Upon entry into a cell, the genome translocates to the nucleus where viral gene expression is initiated. The HCMV genome is 235 kbp in size and has the potential to express 751 proteins during lytic replication [2]. The expression occurs in temporal phases defined as immediate early (IE), early (E), early-late (E-L), and late (L). HCMV proteins play diverse roles during infection including regulation of viral gene expression, replication of genomes, and assembly of capsids. Most of the processes occur within a viral replication compartment located in the nucleus. Replication culminates with the packaging of synthesized viral genomes into capsids followed by nuclear egress.

Efficient HCMV replication also requires manipulation of cellular processes that occur within the nucleus. Examples include manipulation of cellular gene expression, cell cycle regulation and the intrinsic immune response. Several HCMV proteins have a global impact on gene expression achieved using a variety of mechanisms. For instance, HCMV IE1 is necessary and sufficient to promote the nuclear localization of cytosolic STAT3 and alter STAT3-dependent gene expression [3]. Alternatively, HCMV pUL29/28 contributes to stabilizing the tumor suppressor protein p53 and disrupting p53-dependent gene expression [4]. Numerous viral proteins also target cell cycle regulators. Examples include pp71-dependent degradation [5] and pUL97 kinase-mediate phosphorylation [6] of the retinoblastoma protein, Rb. These changes contribute to the activation of pro-proliferative E2F transcription factors (Reviewed in [7]). Finally, viral proteins disarm the cellular intrinsic immune response by disrupting promyelocytic leukemia (PML) nuclear bodies (PML-NB) (Reviewed in [8]), and this is accomplished, in part, by pp71-dependent degradation of hDaxx [9]. Cumulatively, viral proteins manipulate diverse processes resulting in a nuclear environment that supports efficient viral replication.

The objective of our studies is to uncover additional nuclear processes that are being manipulated during infection. HCMV proteins manipulate processes using diverse mechanisms that predominantly result in changes in protein expression, localization, stabilization and modifications. To identify additional processes, we have quantified the HCMV-induced nuclear proteome during infection.

2 Materials and Methods

2.1 Biological reagents

MRC-5 embryonic lung fibroblasts, ARPE19 epithelial cells and telomerase-immortalized human foreskin fibroblast cells (Tel12) [10] were propagated in Dulbecco's modified Eagle medium supplemented with 7% fetal bovine serum (FBS) (Life Technologies) and 1% penicillin-streptomycin (Life Technologies) in 5% CO₂ at 37°C. The bacterial artificial chromosome (BAC)-derived HCMV isolate TB40/E was utilized for infections [11]. Virus was propagated in ARPE19 epithelial cells. Stocks were prepared by centrifugation through

a sorbitol cushion (20% D-sorbitol, 50 mM Tris-HCl [pH 7.2], 1 mM MgCl₂) and suspended in serum-free DMEM. Viral titers were determined by using an immunofluorescence assay to quantify IE1-expressing cells as previously described [4]. For MS analyses and validation experiments, MRC-5 cells were infected at a multiplicity of infection (MOI) of 5.0 IU/cell. For small interfering RNA (siRNA) studies, siRNAs targeting KPNA2 (On-Target Smart pool; GE Healthcare), KPNA3 (On Target Smart pool; GE Healthcare), or scrambled control (GE Healthcare) were transfected into Tel12 cells using Lipofectamine 2000 (Life Technologies). At 24 hr post-transfection, cells were infected at 0.25 IU/cell and harvested at various time points for subsequent analyses.

2.2 SILAC labeling

SILAC media was made using SILAC DMEM (ThermoScientific) supplemented with 10% dialyzed FBS (Sigma) and 1% streptomycin/penicillin in 5% CO₂ at 37°C. The medium was then divided and supplemented with ¹³C₆¹⁵N₄ L-arginine (Sigma) and ¹³C₆, ¹⁵N₂-L-lysine (Sigma) or normal L-arginine (Sigma) and L-lysine (Sigma) to produce heavy (H) or light (L) SILAC medium, respectively. MRC-5 cells were propagated in parallel using H or L SILAC media for 8 cell doublings with media replaced every 24 h. Approximately 10⁷ H-labeled MRC-5 cells were infected with TB40/E at an MOI of 5.0 IU/cell. An equivalent number of L-labeled cells were mock-infected as control. The cells were incubated at 37°C for 2 hr to allow virus to adsorb followed by the addition of pre-warmed L or H medium.

2.3 Protein sample preparation

At 24 hours post infection (hpi), mock-infected L-labeled MRC-5 cells and H-labeled HCMV-infected cells were collected and washed with ice-cold phosphate buffered saline (PBS). Equal numbers of L and H cells were mixed and pelleted by centrifugation at 500xg for 5 min. Cells were then suspended in an Igepal buffer (10mM HEPES, 10mM KCl, 1.5 mM MgCl₂, 0.5mM DTT, 0.5% Igepal) with EDTA-free protease inhibitor cocktail (Roche). The combined cells were then incubated on ice for 5 min followed by gentle mixing. The nuclei were pelleted by centrifugation at 300xg for 5 minutes, and the cytosol was transferred to a fresh microcentrifuge tube. The nuclei pellet was resuspended and washed in the hypotonic buffer with gentle mixing and pelleted again by centrifugation at 300 x g for 5 minutes. The resulting supernatant was added to the cytosol fraction. Pelleted nuclei were lysed using UPX protein extraction buffer (Expedeon) with the addition of protease inhibitor cocktail (Sigma). Protein concentration in nuclear extracts was determined using Bradford Assay Reagent (Sigma). Protein samples were fractionated by mass into 12 liquid-soluble fractions using gel-eluted liquid-fraction entrapment electrophoresis or GELFrEE [12] (Expedeon). Briefly, 400 µg of nuclear extract in 150 µl sample volume was denatured at 95°C for 5 minutes and the sample was loaded onto a 10% Tris-Acetate gel column cartridge (Expedeon). For resolution of nuclear proteins, a constant voltage was applied between the anode and cathode reservoirs. Proteins were eluted from the column at multiple time intervals. The resulting twelve fractions were collected and proteins precipitated by chloroform-methanol extraction.

2.4 Protein digestion and mass spectrometric analysis

For each of the 12 fractions, proteins were resuspended in 50 mM ammonium bicarbonate. Samples were reduced with 10 mM dithiothreitol (DTT) for 30 min at 37°C and alkylated with 50 mM iodoacetamide. Excess alkylating agent was removed by the addition of 10 mM DTT. Samples were digested with Trypsin gold, mass spectrometry grade (Promega) at an enzyme-to-substrate ratio of 1:50 overnight at 37°C. Digestion reactions were stopped by the addition of trifluoroacetic acid to a final concentration of 0.1%. Samples were then desalted and processed by Zip-Tip C18 columns (Millipore). For each of the 12 fractions, approximately 5 µg of tryptic peptides were loaded via autosampler (AS-2; Eksigent Technologies Inc.) onto a 100 µm ID trapping column that was hand-packed with 2.5 cm of 5 µm, 200Å C18 (Magic C18 AQ) over 6 minutes in 2% acetonitrile with 0.1% formic acid. Peptides were eluted from the trapping column onto a 75 µm ID analytical column that was hand-packed with 10 cm of 3 µm, 200Å C18 (Magic C18 AQ). Elution occurred over a 180 minute 2–98% buffer B gradient at a flow rate of 300 nL/min delivered by a NanoLC 2D HPLC pump (AB Sciex Eksigent). Buffer A was 0.1% formic acid in H₂O and buffer B was 0.1% formic acid in acetonitrile. The HPLC effluent was directly coupled to the nanoelectrospray ionization source of an LTQ-Orbitrap Velos hybrid mass spectrometer (ThermoScientific). Precursor ion scans were collected with one internal lock mass at a resolution setting with a resolution of 60,000 for full MS followed by data-dependent and targeted MS/MS acquisitions of the 10 most abundant ions. The normalized collision energy was set to 35. Dynamic exclusion, monoisotopic precursor selection, and predicted ion injection time were enabled. This was done in technical duplicate for three independent biological experiments.

2.5 Protein quantification and identification

Protein identification and quantification were performed using MaxQuant 1.2.2.5 [13] and UniProtKB Homo sapiens reference proteome database containing 70,136 canonical and isoform sequences (retrieved February 2013) through MaxQuant's built-in Andromeda search engine. HCMV reference proteome for strains TB40/E, FIX, AD169, and Toledo was added and used for HCMV peptide identification. Default parameters in MaxQuant were used wherever applicable. Briefly, variable modifications included Lys8 and Arg10, protein N-terminus acetylation, and oxidized methionine. Carbamidomethylated cysteine was the only fixed modification specified. Full trypsin specificity was selected as digestion mode and maximum missed cleavages were set to 2. Peptides with lengths of a minimum of 7 amino acids were considered, with both the peptide and protein false discovery rate (FDR) set to 1%. Precursor mass tolerance was set to 20 ppm for the first search and 4.5 ppm for the main search. Product ions were searched with a mass tolerance of 20 ppm. Protein identification required a minimum of two peptides with at least one razor or unique peptide. Relative ratio quantification was performed using quantities of unique and razor peptides and required a minimum of two peptides. Identified proteins that could be reconstructed from a set of peptides are “grouped” and termed protein groups. The top matched or leading protein in a protein group is defined as the protein with the greatest number of identified peptides within the group, and these are listed in Tables 1 and S1. Protein groups marked as contaminant, reverse, or “identified by site only” in MaxQuant results were discarded. Protein groups identified by MaxQuant were imported to Perseus version 1.4.1.3 for

statistical analysis. Normalized protein group quantity relative ratios were first converted to log base 10, and significantly changed protein groups were identified using an outlier significance score for log protein ratios, Significance A calculation in Perseus with default parameters (threshold value 0.05). Significance B was then calculated to correct for the biased statistical spread of highly abundant proteins. MaxQuant protein group ID, peptide ID, and parameter files have been deposited to ProteomeXchange consortium.

2.6 Gene ontology analysis

Protein groups identified by MaxQuant were imported into ProteinCenter (Proxeon Bioinformatics, Odense, Denmark) for Gene Ontology Biological Process (GOBP) enrichment by statistical analysis. ProteinCenter software utilizes a database that includes all major protein databases (13 protein databases (identifiers), 34 gene (identifiers), 2 gene annotation, 3 domain, 3 core annotation, 4 sequence, 3 GO annotation). Annotation categories disproportionately represented in the dataset were determined by statistical analysis and characterized as over- or under-represented. A p-value for the number of occurrences in the dataset was then estimated based on a significance threshold (threshold value 0.05) set prior to analysis. GO terms were clustered reducing functional redundancies based on semantic similarity and p-values using REVIGO [14] to identify HCMV-targeted biological processes.

2.7 Western blot analysis, immunofluorescence and quantitative PCR

Western blot analysis of nuclear fractions from infected MRC-cells was performed as follows. MRC-5 cells were infected with TB40/E at MOI of 5 IU/cell. At 24, 48 and 72 hpi, unlabeled MRC-5 cells were harvested and nuclear extracts were prepared as described above. Nuclear proteins were resolved on an SDS 10% PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked using 5% skim milk in PBS with Tween20 (PBST) and probed using various antibodies in 1% BSA in PBST. The following primary antibodies were used for Western blot or immunofluorescence analysis: mouse anti-IE1 and mouse anti-UL38 (generously provided by Tom Shenk, Princeton University), mouse anti-lamin B1 (clone 119D5-F1, Santa Cruz), mouse anti- α -tubulin (clone DM1A, Santa Cruz), mouse anti-fibroblast surface protein (clone 1B10, Abcam), rabbit anti-KPNA2 (clone ab70160, Abcam), goat anti-KPNA3 (clone ab6038, Abcam), rabbit anti-KPNB1 (clone 3873, Cell Signaling), mouse anti-GAPDH (clone 0411, Santa Cruz), mouse anti-JUNB (clone C-11, Santa Cruz), mouse anti-UHRF1 (clone H-B, Santa Cruz), rabbit anti-MTA2 (clone H-170, Santa Cruz), rabbit anti-MBD2 (clone H-70, Santa Cruz). Secondary antibodies included anti-goat-horseradish peroxidase (HRP), anti-rabbit-HRP, and anti-mouse-HRP (Jackson ImmunoResearch). For validation studies, anti-rabbit-Alexa Fluor 488, anti-mouse-Alexa Fluor 488, anti-goat Alexa Fluor 488 (Life Technologies) conjugates were used for detection and quantification using Typhoon Trio Variable Mode Imager (GE Healthcare). Validation data are representative of at least 2 independent experiments and presented as the mean $\pm$ the standard error of the mean. For immunofluorescence, cells were fixed and stained using an anti-IE1 primary antibody followed by Alex Fluor 586. Slides were mounted in SlowFade Gold (Invitrogen) with DAPI reagent and visualized on a Nikon Ti Eclipse confocal microscope. The areas of nuclei were quantified using NIS Elements software (Nikon) from 20 cells at each time point. Viral DNA and RNA expression from

infected cells were determined using quantitative PCR or RT-PCR as previously described [4]. Data are representative of at least 2 independent experiments, and values are given as the mean of replicate experiments $\pm$ the standard error of the mean.

3 Results

3.1 Quantitative proteomic analysis of the host nuclear proteome during infection

HCMV infection has a profound impact on the basic physiology of the nucleus including reorganization of domains as well as increased size (Fig. 1A and B) and protein content (Fig. 1C). These changes are continuous over time culminating with the packaging of viral genomes into newly assembled viral capsids and nuclear egress. Our goal was to identify HCMV-manipulated cellular processes occurring early during infection that likely contribute to efficient replication. We have examined changes at 24 hpi to generate a snapshot of the nuclear proteome coinciding with the onset of HCMV viral DNA synthesis and prior to an increase in the size of the nucleus. We have used stable isotope labeling of amino acids in cell culture (SILAC) in conjunction with a nanoLC-MS/MS platform to identify and quantify changes in nuclear proteins during lytic infection in primary fibroblasts. The workflow is presented in Figure 1D. Low passage MRC-5 fibroblasts were cultured in either light or heavy media until greater than 98% of peptides identified in the population incorporated heavy isotope-labeled amino acids (Fig. 1S). We infected heavy-labelled cells at a multiplicity of 5 infectious units (IU)/cells using the HCMV clinical isolate TB40/E. At 24 hpi, mock light and infected heavy cells were mixed at a 1:1 ratio and the sample was enriched for cellular nuclei. Bright field images and Western blot analyses were completed to control for the efficiency of enrichment of the uninfected and infected nuclei (Fig. 1E). Nuclear proteins were resolved using gel-eluted liquid fraction entrapment electrophoresis (Fig. 1F), precipitated, and subjected to tryptic digestion. Each fraction was analyzed in duplicate by nanoLC-MS/MS from three independent biological replicate experiments.

The resulting spectra were combined and analyzed using MaxQuant software. To prevent redundancy, protein groups were identified in MaxQuant based upon peptide evidence including the presence of unique peptides. Our studies identified 1281 cellular protein groups (Tables 1 and S1) and 36 viral proteins (Table 2) associated with nuclei at 24 hpi. Additionally, we observed several cytosolic proteins in our dataset. The identification of these proteins can most likely be attributed to our nuclei enrichment method. Statistical significance of normalized ratios was used to identify differences in protein groups between mock and HCMV-infected proteomes (Table 1). Significance A identified 77 protein groups with 32 increased or 45 decreased ratios in comparison to all quantified groups. Significance B corrected for bias in the statistical spread of unregulated proteins as a result of inherent differences in protein abundance, and identified 28 proteins. Together, these data demonstrate that HCMV infection is altering the nuclear proteome at early times of infection.

3.2 HCMV infection alters diverse cellular processes

To identify cellular processes, we completed a gene ontology (GO) enrichment analysis using the software tool, ProteinCenter. Protein identity and quantitative information was

taken directly from MaxQuant and used to determine whether a biological process was either under- or over-represented disproportionately in the data set. A p-value was calculated for each process identified. HCMV infection induced changes in diverse processes including both statistically under- (Table S2) and over-represented differences (Table S3) at 24 hpi. We clustered redundant GO biological processes based upon similarity and significance to assist in identifying functionally relevant changes as well as effectively presenting the complex dataset. These data are presented in Figure 2. The predominant processes suppressed by HCMV infection include immune response, cation transport, chemical homeostasis and neurological system process (Fig. 2A). Immune response includes changes in PML, heat shock protein HSPA1A and tubulin beta-chain TUBB protein (Table 1). In contrast, the top biological processes induced by infection include mRNA metabolism, RNP complex biogenesis, heterocycle and cyclic compound metabolism, transport, and cell cycle (Fig. 2B). mRNA metabolism includes the process of gene expression and proteins changing in this category are the E3 ligase UHRF1, transcription factor JUNB, kinase CDK9 and mediator component MED11 (Table 1). Infection also induced an increase in nuclear transport with increases in a subset of nuclear transport factors, karyopherins. Altered protein levels were observed for KPNA2 and KPNA3 (Table 1). Collectively, our studies identified HCMV-manipulated biological processes with the most prominent changes being increased mRNA metabolism and decreased immune response.

To validate a subset of heavy-to-light ratios, we completed Western blot analysis using protein-specific antibodies. Nuclei were isolated from mock and HCMV-infected cells at 24 hpi and subjected to Western blot analysis. We evaluated changes in UHRF1 and JUNB, involved in gene expression as well as KPNA2 and KPNA3, involved in transport (Table 1). Protein levels were determined using fluorescently-labeled secondary antibodies and detection by a fluorescence imager. Quantification was completed using a minimum of two biological replicates and normalized to the levels of lamin B. We observed increased levels of UHRF1, JUNB, KPNA2 and KPNA3 at 24 hpi compared to mock (Fig. 3A). The fold-differences detected in these experiments are consistent with the calculated heavy-to-light ratios (Fig. 3A, Table 1). We also evaluated proteins that did not significantly change including the transport factor KPNB1 and transcriptional regulators MTA1 and MBD2. Infection resulted in small differences in expression levels of these factors as determined by Western blot analysis and this matches their heavy-to-light ratios (Fig. 3A). Together, these data demonstrate that the SILAC ratios reliably indicate relative abundances of selected proteins identified in the proteomic dataset.

3.3 HCMV differentially regulates the cellular nuclear import machinery

To assess the biological relevance of our studies, we evaluated the impact of nuclear import factors on HCMV infection. The classical nuclear import mechanism is one of several import mechanisms and involves cytosolic association of a cargo protein with one of seven KPNA adaptors and KPNB1 followed by translocation through the nuclear pore. During infection, we detected significant changes in KPNA2 and 3 levels (Table 1). Next, we evaluated changes in KPNA2 and 3 as well as KPNB1 during the course of infection. Western blot analysis was completed using nuclear extracts collected at varying times post infection. We observed increased steady-state levels for all three import factors out to 72 hpi

(Fig. 3B). To determine whether the increase is dependent on expression of viral genes, we repeat the infections using wild-type inoculum or UV-inactive inoculum (Fig. 3C). HCMV IE1 RNA expression was disrupted by UV treatment as determined by RT-qPCR (Fig. 3C). In these experiments, we failed to detect changes in expression following UV treatment suggesting that the induction is dependent upon HCMV gene expression (Fig. 3C). Next, we were interested in determining whether KPNA2 and 3 adaptor proteins have a functional impact on infection. We transfected gene-specific siRNAs into fibroblasts which resulted in substantial reductions in KPNA2 and 3 protein levels as compared to control siRNA in both mock and HCMV infected cells (Fig 3D). Disruption of either KPNA resulted in a 50% reduction in viral genome levels at 72 hpi compared to the control. We observed an additive effect when both factors were disrupted resulting in a 90% reduction (Fig. 3D). The size of nuclei were not altered between control and KPNA siRNA-treated samples at 72 hpi (data not shown). Our data demonstrate that HCMV infection induces expression of a subset of nuclear import factors early during infection and this event is necessary for efficient viral genome synthesis.

4 Discussion

HCMV infection has a profound impact on the physiology of nuclei during the course of viral replication. The goal of this study was to identify nuclear processes that are manipulated early during infection by quantifying changes in the nuclear proteome. We have used SILAC-based quantitative proteomics to identify virally-induced changes in nuclear protein content. We uncovered 77 cellular proteins that are significantly differentially expressed from a total of 1281 cellular protein groups identified. In comparison to studies by Weekes *et al.* [15], 62 of the 77 proteins (Table 1) were identified in both studies with 28 of 32 proteins observed to increase and 28 of 45 proteins observed to decrease. The identification of additional proteins is likely the result of nuclei enrichment. In addition, we observed 36 viral proteins to be associated with the nucleus at 24 hpi including several of unknown functions. Using the total proteins identified as well as the quantitative information, we have defined a subset of cellular processes that are manipulated early during infection. Processes suppressed by HCMV include immune response, cation transport, chemical homeostasis and neurological system process while those induced include mRNA metabolism, RNP complex biogenesis, heterocycle and cyclic compound metabolism, transport, and cell cycle. These studies have identified several previously unknown cellular processes that are altered early during HCMV infection.

We observed that infection significantly up regulates the process of nuclear transport. We identified and validated increased levels of the import factors, KPNA2 and 3 during infection. In general, the classical import mechanism involves binding of an NLS-containing cargo protein to one of seven KPNA factors and KPNB1. KPNA expression varies between cell types and differentiation states. Several HCMV proteins that utilize an NLS-mediated import mechanism have been shown to bind to KPNA factors (Reviewed in [16]). We have demonstrated that the increased levels of KPNA2 and 3 are dependent upon HCMV gene expression and that disrupting the event is inhibitory to the viral DNA synthesis. Other viruses exploit the differential expression of KPNA. For example, KPNA expression influences influenza A virus tropism by increasing nuclear import as well as regulating viral

polymerase activity [17]. It is conceivable that HCMV induces KPNA expression to promote increased import of viral proteins and future studies will test this hypothesis. Our studies identified a subset of KPNA factors that are important for HCMV replication.

Chronic HCMV infection has been associated with diverse pathologies with the most recent being cancer, albeit controversial. The virus exhibits oncomodulatory properties by expressing proteins that manipulate cancer-associated processes (Reviewed in [18]). Our studies are consistent with this having observed increased manipulation of cell cycle-related processes and mRNA metabolism while decrease processes in immune responses. In addition, we have identified several proteins that may contribute to oncomodulatory properties. Examples include KPNA2 which is a biomarker for several cancers and correlates with increased cell proliferation [19]. The epigenetic regulators UHRF1 and DNMT1, whose expression is also significantly up regulated by 24 hpi, promote cell growth [20] and function in a coordinated fashion [21]. We observed that infection induces increased levels of the pro-proliferative transcription factor JUNB (Reviewed in [22]). Finally, we identified several RNA helicases to be significantly induced early during HCMV infection. This includes DDX21 which is highly expressed in several cancers [23] and contributes to coordinating transcription with ribosomal RNA processing [24]. Overall, our studies have identified new cellular proteins as well as processes that are manipulated by HCMV early during infection and represent possible targets of antiviral therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HCMV	human cytomegalovirus
IE	immediate early gene
E	early gene
E-L	early-late gene
L	late gene
IU	infectious units
hpi	hours post infection
UBX	Universal Protein Extraction
GELFrEE	gel-eluted liquid-fraction entrapment electrophoresis

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Figure 1. Characterization of HCMV-induced changes in the host cell nuclear proteome early during infection

(A) Immunofluorescence analysis of nuclei from mock- and HCMV-infected cells at 24, 48 and 72 hpi. MRC-5 cells were infected with the HCMV clinical isolate TB40/E expressing the mCherry protein. Cells were fixed and stained using DAPI (blue) and an antibody against HCMV IE1 with an Alex Fluor 568-conjugated secondary antibody (red). Scale bars 10 μ m. (B) The areas of nuclei were quantified using the area function on Nikon NIS Elements software from 20–50 cells at each time point from two biological replicates. (C) Nuclear extracts enriched from mock (m) and HCMV-infected MRC-5 cells at the indicated times post infection. Lysates were loaded based on cell equivalents, resolved by SDS 4–20% PAGE gel and visualized by silver stain. (D) Schematic of the experimental workflow used to quantify changes in the nuclear proteome. (E) Brightfield images of HCMV-infected cells prior to (left) and after enrichment of nuclei (right). Western blot analysis of the nuclear (nuc) and cytoplasmic (cyt) extracts using antibodies to specific markers for each fraction. (F) Nuclear extract from a 1:1 mixture of mock and HCMV-infected cells was resolved by 10% Tris-acetate gel cartridge using gel-eluted liquid-fraction entrapment electrophoresis (GEL-FREE). An aliquot of fractions 4–12 were resolved by SDS 4–20% PAGE gel and visualized by silver stain.

Figure 2. Functional analysis of proteins quantified within nuclei of HCMV-infected cells
Proteins were classified by gene ontology annotation based on Gene Ontology Biological Process (GOBP) using ProteinCenter software. The top 100 under- (A) and over-represented (B) GO processes were clustered based on p-values using REVIGO. Specific GO processes and associated values are presented in Tables S2 and S3.

Cellular proteins identified to be significantly altered during HCMV infection in fractions enriched for cellular nuclei

Table 1

UniProt Accession	Gene Name	Protein Name	Ratio H/L Normalized	Significance A (p-value)	Significance B (p-value)	Unique Peptides	Total Peptides	Coverage [%]	Posterior Error Probability
Q96T88	UHRF1	E3 ubiquitin-protein ligase UHRF1	5.22	1.41E-07	9.45E-06	11	11	15.3	1.04E-159
Q5SX90	GDI2	Rab GDP dissociation inhibitor beta	4.43	5.67E-06	1.37E-04	11	11	36.3	0.00E+00
P52292	KPNA2	Importin subunit alpha-1	4.16	1.42E-04	3.91E-06	14	14	40.5	0.00E+00
P17275	JUNB	Transcription factor jun-B	3.51	8.00E-07	3.32E-05	2	2	8.4	9.79E-14
P61026	RAB10	Ras-related protein Rab-10	3.45	2.76E-02	6.78E-02	3	4	18.0	7.73E-43
Q14807	KID	Kinesin-like protein KIF22	2.99	1.02E-03	6.03E-03	3	3	4.8	7.97E-05
Q9Y5K6	CD2AP	CD2-associated protein	2.67	2.29E-03	1.09E-02	4	4	5.9	1.75E-16
Q8N1G2	CMTR1	Cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	2.63	1.85E-03	9.30E-03	3	3	3.4	1.08E-04
Q03111	MLLT1	Protein ENL	2.61	3.43E-03	1.46E-02	2	2	4.3	3.21E-08
Q9H501	ESF1	ESF1 homolog	2.60	4.60E-03	1.81E-02	2	2	2.9	1.27E-34
Q13206	DDX10	Probable ATP-dependent RNA helicase DDX10	2.47	1.11E-02	3.47E-02	7	7	9.3	3.17E-41
P50750	CDK9	Cyclin-dependent kinase 9	2.47	7.99E-03	2.72E-02	4	5	10.6	1.68E-10
Q9P086	MED11	Mediator of RNA poly. II transcription subunit 11	2.45	3.76E-03	1.56E-02	2	2	23.1	7.94E-11
O75182	SIN3B	Paired amphipathic helix protein Sin3b	2.44	8.80E-03	2.92E-02	3	3	3.9	8.47E-25
Q59GX2	SLC2A1	Solute carrier family 2	2.40	1.09E-02	3.41E-02	2	2	3.5	2.68E-10
J3QRU1	YES	Tyrosine-protein kinase Yes	2.35	3.24E-03	1.40E-02	4	5	8.4	6.52E-11
O00505	KPNA3	Importin subunit alpha-4	2.35	1.50E-02	4.32E-02	10	13	37.4	4.43E-127
O75152	ZC3H1A	Zinc finger CCH domain-containing protein 11A	2.35	1.16E-02	3.57E-02	10	10	16.5	1.84E-141
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	2.33	1.10E-02	3.44E-02	4	4	2.7	7.14E-09
Q9NR30	DDX21	Nucleolar RNA helicase 2	2.31	1.01E-02	1.71E-03	31	33	46.9	0.00E+00
Q9H0U9	TSPYL1	Testis-specific Y-encoded-like protein 1	2.28	1.62E-02	4.57E-02	6	6	21.3	1.27E-54
Q9NYY0	TERF2IP	Telomeric repeat-binding factor 2-interacting protein 1	2.23	1.29E-02	3.86E-02	6	6	30.3	7.17E-291
O95983	MBD3	Methyl-CpG-binding domain protein 3	2.23	7.75E-03	2.66E-02	3	3	10.3	1.99E-79
Q86YP4	GATAD2A	Transcriptional repressor p66-alpha	2.18	1.74E-02	4.82E-02	7	8	17.5	4.50E-69
E7ENF1	C12orf43	Uncharacterized protein C12orf43	2.15	2.70E-02	6.66E-02	2	2	9.9	4.77E-05
P08107	HSPA1A	Heat shock 70 kDa protein 1A/1B	2.09	2.08E-02	4.73E-03	1	14	29.3	4.99E-203
O00148	DDX39A	ATP-dependent RNA helicase DDX39A	2.07	2.78E-02	7.13E-03	6	18	53.4	0.00E+00

UniProt Accession	Gene Name	Protein Name	Ratio H/L Normalized	Significance A (p-value)	Significance B (p-value)	Unique Peptides	Total Peptides	Coverage [%]	Posterior Error Probability
P51531	SMARCA2	Probable global transcription activator SNF2L2	2.06	1.52E-02	4.36E-02	2	5	3.0	3.19E-42
Q9BZK7	TBL1XR1	F-box-like/WD repeat-containing protein TBL1XR1	1.97	3.37E-02	9.32E-03	10	15	39.7	0.00E+00
Q9Y2K7	KDM2A	Lysine-specific demethylase 2A	1.95	1.50E-02	4.31E-02	7	7	8.6	3.29E-21
Q9Y2R4	DDX52	Probable ATP-dependent RNA helicase DDX52	1.87	3.84E-02	8.65E-02	11	12	29.4	0.00E+00
Q9GZR7	DDX24	ATP-dependent RNA helicase DDX24	1.85	3.11E-02	8.34E-03	17	17	25.6	4.16E-190
P29590	PML	Protein PML	0.94	3.53E-03	1.32E-02	10	10	13.5	4.29E-65
P53396	ACLY	ATP-citrate synthase	0.85	3.70E-02	7.47E-02	5	5	6.0	3.57E-42
E7EUT5	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.69	3.81E-03	5.59E-04	8	9	34.9	0.00E+00
P07437	TUBB	Tubulin beta chain	0.64	6.85E-03	1.27E-03	6	12	43.5	8.64E-134
P48643	CCT5	T-complex protein 1 subunit epsilon	0.60	1.22E-03	6.11E-03	4	4	11.1	7.15E-73
P40227	CCT6A	T-complex protein 1 subunit zeta	0.58	5.01E-03	1.71E-02	7	7	15.4	1.79E-125
Q02539	HIST1H1A	Histone H1.1	0.56	3.49E-02	7.15E-02	4	11	42.3	9.62E-139
P49368	CCT3	T-complex protein 1 subunit gamma	0.53	2.49E-03	1.03E-02	8	8	16.5	2.01E-134
P78371	CCT2	T-complex protein 1 subunit beta	0.52	1.79E-03	8.06E-03	5	5	14.0	1.92E-60
P25705	ATP5A1	ATP synthase subunit alpha, mitochondrial	0.52	1.28E-02	3.39E-02	5	5	13.0	9.19E-48
Q12797	ASPH	Aspartyl/asparaginyl beta-hydroxylase	0.51	2.53E-02	5.63E-02	9	9	15.8	7.15E-185
Q9NUQ6	SPATS2L	SPATS2-like protein	0.51	3.67E-02	7.42E-02	7	7	15.5	4.22E-44
Q4LE64	NUMA1	NUMA1 variant protein	0.51	4.96E-03	8.08E-04	44	44	29.4	0.00E+00
P23497	SP100	Nuclear autoantigen Sp-100	0.50	5.72E-07	2.45E-05	2	4	5.1	3.97E-88
P06576	ATP5B	ATP synthase subunit beta, mitochondrial	0.48	2.46E-02	5.51E-02	6	6	15.5	5.02E-177
Q9NZI8	IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1	0.46	3.77E-02	7.57E-02	5	5	12.8	5.10E-109
P13639	EEF2	Elongation factor 2	0.39	3.87E-04	2.65E-03	11	12	20.6	2.01E-206
F8W930	IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2	0.35	1.08E-02	3.00E-02	7	8	17.9	7.61E-105
Q00341	HDLBP	Vigilin	0.35	9.94E-03	2.82E-02	2	11	12.7	9.90E-120
O00425	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3	0.35	5.37E-03	1.80E-02	8	9	21.9	1.29E-189
Q92522	H1FX	Histone H1x	0.34	1.13E-03	5.76E-03	3	3	15.5	4.25E-94
Q07065	CKAP4	Cytoskeleton-associated protein 4	0.33	9.23E-03	1.93E-03	34	34	60.0	0.00E+00
P01040	CSTA	Cystatin-A	0.31	5.29E-05	6.30E-04	3	3	42.9	1.22E-32
Q99715	COL12A1	Collagen alpha-1(XII) chain	0.28	1.03E-02	2.89E-02	14	14	6.0	3.00E-165
Q9P2E9	RRBP1	Ribosome-binding protein 1	0.24	6.34E-05	1.69E-06	22	22	20.8	0.00E+00

UniProt Accession	Gene Name	Protein Name	Ratio H/L Normalized	Significance A (p-value)	Significance B (p-value)	Unique Peptides	Total Peptides	Coverage [%]	Posterior Error Probability
Q96D15	RCN3	Reticulocalbin-3	0.24	2.99E-04	2.20E-03	4	4	17.4	5.11E-17
P31944	CASP14	Caspase-14	0.16	3.40E-03	1.29E-02	4	4	17.8	2.33E-85
P13674	P4HA1	Prolyl 4-hydroxylase subunit alpha-1	0.16	1.11E-07	1.90E-10	15	15	37.1	0.00E+00
Q15113	PCOLCE	Procollagen C-endopeptidase enhancer 1	0.16	1.94E-06	1.15E-08	7	7	21.8	1.27E-71
P05109	S100A8	Protein S100-A8	0.16	1.82E-06	5.60E-05	4	4	38.7	1.20E-15
P07237	ERBA2L	Protein disulfide-isomerase	0.15	5.14E-08	6.33E-11	16	16	38.8	2.09E-273
O43175	PGDH3	D-3-phosphoglycerate dehydrogenase	0.15	3.90E-05	5.06E-04	2	2	4.5	1.85E-16
Q9NZT1	CALML5	Calmodulin-like protein 5	0.11	1.54E-16	3.94E-12	4	4	30.8	1.03E-52
P02452	COL1A1	Collagen alpha-1(I) chain	0.09	5.67E-08	7.28E-11	33	33	39.2	0.00E+00
P08123	COL1A2	Collagen alpha-2(I) chain	0.08	7.98E-11	4.40E-08	17	17	17.7	2.88E-181
P81605	DCD	Dermcidin	0.08	2.41E-11	1.88E-08	3	3	20.7	7.08E-14
P14923	JUP	Junction plakoglobin	0.08	4.26E-10	1.45E-07	9	18	29.9	0.00E+00
P06702	S100A9	Protein S100-A9	0.07	1.44E-16	3.76E-12	3	3	30.7	2.38E-99
P15924	DSP	Desmoplakin	0.07	8.87E-13	1.81E-09	39	39	14.7	0.00E+00
AZGP1	AZGP1	Zinc-alpha-2-glycoprotein	0.06	4.67E-15	4.40E-11	3	3	15.8	7.30E-08
Q96P63	SERPINB12	Serpin B12	0.06	1.56E-09	3.64E-07	5	5	14.4	1.06E-23
C9JF17	APOD	Apolipoprotein D	0.05	5.33E-04	3.34E-03	4	4	18.6	1.11E-07
Q13835	PKP1	Plakophilin-1	0.05	2.07E-06	6.15E-05	2	2	2.9	1.02E-06
Q01469	FABP5	Fatty acid-binding protein, epidermal	0.04	1.19E-22	1.90E-16	2	2	13.3	4.14E-25
O14556	GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	0.02	2.51E-27	9.64E-20	1	2	4.4	4.27E-11

Table 2

HCMV proteins identified in fractions enriched for cellular nuclei

UniProt Accession	Gene Names	Protein Names	Unique Peptides	Total Peptides	Coverage [%]	Posterior Error Probability
A8T770	UL13	Protein UL13	5	5	16.5	1.6E-64
A8T788	UL25	Tegument protein UL25	15	15	33.5	1.1E-278
P16762	UL26	Tegument protein UL26	2	2	11.7	3.3E-48
A8T791	UL27	Protein UL27	2	2	4.6	1.8E-13
E7DVH9	UL29	Protein UL29	9	20	31.8	0.0E+00
A8T797	UL31	UL31	3	3	7.9	1.4E-49
A8T798	UL32	Tegument protein pp150	17	17	19.1	0.0E+00
A8T7A3	UL35	Tegument protein UL35	6	6	14.5	1.5E-59
A8T7A4	UL36	Tegument protein vICA	14	14	39.1	2.0E-155
D2K4U0	UL37	Envelope glycoprotein UL37	2	2	5.5	1.3E-74
Q6RXH4	UL44	DNA polymerase processivity factor	17	17	49.4	0.0E+00
A8T7B8	UL45	Ribonucleotide reductase subunit 1	3	3	3.5	3.0E-18
Q6RXH2	UL46	Capsid triplex subunit 1	7	7	32.1	3.7E-176
D3YRY6	UL48	Large tegument protein	3	3	1.7	8.2E-12
A8T7D3	UL54	DNA polymerase	24	24	25.4	4.6E-163
A8T7D7	UL57	Single-stranded DNA-binding protein	16	16	16.4	3.9E-130
P16749	UL69	mRNA export factor ICP27 homolog	6	6	12.1	6.5E-38
A8T7F7	UL80	Capsid maturation protease	5	5	19.4	8.0E-37
D3YS06	UL82	Tegument protein pp71	16	16	41.4	0.0E+00
A8T7G1	UL83	Tegument protein pp65	25	25	66.8	0.0E+00
D2K4X7	UL84	Protein UL84	4	4	7.7	3.2E-33
Q6RXF2	UL85	Capsid triplex subunit 2	7	7	29.7	2.2E-146
A8T7G6	UL86	Major capsid protein	45	45	47.3	0.0E+00
A8T7H8	UL94	UL94	2	2	4.9	1.9E-08
Q5IV09	UL97	Tegument ser/thr protein kinase	8	8	17.5	3.8E-206
A8T7I4	UL98	Deoxyribonuclease	3	3	8.0	3.8E-22
A8T7I8	UL102	Helicase-primase subunit	2	2	3.5	1.8E-54
A8T7I4	UL112	Protein UL112	3	12	24.5	7.5E-168

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UniProt Accession	Gene Names	Protein Names	Unique Peptides	Total Peptides	Coverage [%]	Posterior Error Probability
A8T7J5	UL114	Uracil-DNA glycosylase	4	4	18.4	6.9E-09
Q6SWY2	UL122	Regulatory protein IE2	1	4	7.1	1.1E-130
Q6SWY1	UL123	Regulatory protein IE1	5	8	20.8	3.4E-176
A8T7N5	UL135	UL135	4	4	17.7	1.3E-07
Q6SWX5	UL148	Membrane protein UL148	4	4	12.7	3.3E-11
A8T7Z5	US22	Tegument protein US22	21	21	39.9	0.0E+00
Q6RXA1	US24	Tegument protein US24	2	2	6.4	7.7E-10
D2K564	TRS1	Tegument protein TRS1	8	8	14.5	5.0E-174