Research Paper

Comprehending a Killer: The Akt/mTOR Signaling Pathways Are Temporally High-Jacked by the Highly Pathogenic 1918 Influenza Virus

Charlene Ranadheera a,b, Kevin M. Coombs a,c,d,⁎, Darwyn Kobasa a,b,⁎⁎

a Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 9J6, Canada
b Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba R3E 3R2, Canada
c Manitoba Centre for Proteomics & Systems Biology, Room 799, 715 McDermot Avenue, Winnipeg, Manitoba R3E 3P4, Canada
d Manitoba Institute of Child Health, John Buhler Research Centre, Room 513, 715 McDermot Avenue, Winnipeg, Manitoba R3E 3P4, Canada

⁎⁎ Correspondence to D. Kobasa, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada.
E-mail addresses: kevin.coombs@umanitoba.ca, (K.M. Coombs), darwyn.kobasa@phac-aspc.gc.ca (D. Kobasa).

A R T I C L E   I N F O

Article history:
Received 15 February 2018
Received in revised form 8 May 2018
Accepted 21 May 2018
Available online 2 June 2018

Keywords:
RNA virus
Virus infection
Host cell alterations
Mass spectrometry
Liquid chromatography
Bioinformatics

A B S T R A C T

Previous transcriptomic analyses suggested that the 1918 influenza A virus (IAV1918), one of the most devastating pandemic viruses of the 20th century, induces a dysfunctional cytokine storm and affects other innate immune response patterns. Because all viruses are obligate parasites that require host cells for replication, we globally assessed how IAV1918 induces host protein dysregulation. We performed quantitative mass spectrometry of IAV1918-infected cells to measure host protein dysregulation. Selected proteins were validated by immunoblotting and phosphorylation levels of members of the PI3K/AKT/mTOR pathway were assessed. Compared to mock-infected controls, >170 proteins in the IAV1918-infected cells were dysregulated. Proteins mapped to amino sugar metabolism, purine metabolism, steroid biosynthesis, transmembrane receptors, phosphatases and transcription regulation. Immunoblotting demonstrated that IAV1918 induced a slight up-regulation of the lamin B receptor whereas all other tested virus strains induced a significant down-regulation. IAV1918 also strongly induced Rab5b expression whereas all other tested viruses induced minor up-regulation or down-regulation. IAV1918 showed early reduced phosphorylation of PI3K/AKT/mTOR pathway members and was especially sensitive to rapamycin. These results suggest the 1918 strain requires mTORC1 activity in early replication events, and may explain the unique pathogenicity of this virus.

1. Introduction

The 1918 influenza pandemic was one of the most devastating infectious disease events of the 20th century, resulting in 20–100 million deaths [56,86]. Although the young and the elderly are usually the most susceptible to influenza A virus (IAV) epidemics and many pandemics, the 1918 pandemic was unusual in that a much larger proportion of healthy young adults succumbed to the infection [86], which has been attributed to a dysfunctional host immune response (cytokine storm) [47,53]. A link between the cytokine storm and IAV-induced pathogenesis and poor clinical outcome has long been appreciated [1,47,49,61,87]. Recent attempts to modulate the cytokine storm, including using lipid-modifying compounds such as sphingosine-1-phosphate [60,61,81] have been only partially successful. Because all viruses are obligate parasites that require a host cell in which to replicate, a more complete and detailed understanding of cell signaling and how IAV induces host protein dysregulation is required (recently reviewed in [87]).

The need to better delineate host responses to IAV infection is further underscored by the nature of the virus. IAV is a small, enveloped virus in the family Orthomyxoviridae, with a genome of 8 negative-sense single stranded RNA segments that encode for at least 15 proteins [39,62]. IAV have enormous genetic plasticity, mediated by nucleotide (genetic drift) and genome segment exchange (genetic shift), changes that control differences in host range and virulence. IAV are serologically categorized by the hemagglutinin (HA) and neuraminidase (NA) proteins, both of which are located in the viral envelope. There are currently 18 recognized HA (H1–H18) and 11 NA (N1–N11) types [62,65,89]. Various anti-viral strategies, including small molecule inhibitors and vaccines, have been developed to combat IAV. However, the virus’ genetic plasticity often leads to resistance rapidly developing to these virus-targeted anti-viral modalities. In addition, because of the virus’ enormous host range, spanning avian, marine mammals and numerous land animals including humans, eradication of the virus is extremely unlikely.

Environmental stressors, including virus infection, induce a number of alterations in a host cell’s transcriptome and proteome. Previous
transcriptomic analyses of cellular responses to IAV have provided some information (for example: [2,28]), including description of innate immune response patterns in macaques infected with the 1918 influenza strain [47]. However, there often is poor concordance between microarray and protein data [2,57,88], partly because mRNA levels cannot provide complete information about extents of post-translational modifications or about levels of effector protein synthesis. Thus, we complemented some of our previous transcriptomic analyses of 1918 virus infection [47] by using a non-biased stable isotope-based quantitative mass spectrometric method to globally assess host proteomic alterations induced by 1918 virus infection in cultured A549 cells.

2. Materials and Methods

2.1. Cells and Viruses

2.1.1. Viruses

All viruses used in this study (Supplementary Table S1), including IAV strain A/South Carolina/1/1918 (H1N1; “1918”), were generated by reverse genetics as previously described [59]. All infectious work was carried out under containment level 4 (CL-4) conditions at the National Microbiology Laboratory in Winnipeg, Canada as outlined in the Health Canada Laboratory Bio-safety Guidelines CL-4 handling procedures (www.hc-sc.gc.ca/pphb-dgspsp/publicat/lbg-ldmbl-96/index.html).

2.1.2. Cells

Human lung A549 cells (American Type Culture Collection # CCL-185) and Madin Darby canine kidney (MDCK) (ATCC # CCL-34) cells were routinely cultured in Dulbecco’s modified MEM (DMEM) supplemented with non-essential amino acids, sodium pyruvate, 0.2% (w/v) glucose, 10% fetal bovine serum (FBS; Invitrogen), and 2 mM L-glutamine as previously described [12]. Virus stocks were generated, and virus titrations were performed by a previously-described high salt/urea double extraction procedure [48] and both fractions frozen at -80°C until further processing. Fractionated samples were probed with antibodies targeting nuclear and cytoplasmic proteins to ensure the method for fractionation was complete (Fig. 5A). Histone H3 is a nuclear protein, Lamin is predominantly found in the nuclear envelope, Actin is typically found in the cytoplasm and tubulin is found in both the nucleus and cytoplasm.

2.2. Mass Spectrometric Sample Preparation and Analysis

Protein content in the various fractions was determined using a BCA™ Protein Assay Kit (Pierce; Rockford, IL) and BSA standards. Samples were then reduced, alkylated and digested with trypsin as previously described [12]. Digested peptides were separated by 2D RP (reversed-phase) high pH – RP low pH peptide fractionation [29,83] and analyzed on a QStar Elite mass spectrometer (Applied Biosystems, Foster City, CA) run in a data-dependent MS/MS acquisition mode as previously described using the manufacturer’s “smart exit” (spectral quality 5) settings [12]. Previously targeted parent ions were excluded from repetitive MS/MS acquisition for 60 x (50 mDa mass tolerance). Protein Pilot 2.0 (Applied Biosystems) software was used for protein identification and quantitation. Raw data files (30 in total for each run) were submitted for simultaneous search using standard SILAC settings for Qstar instruments. Proteins for which at least 2 fully trypsin digested L and H peptides were detected at >99% confidence were used for subsequent comparative quantitative analysis. Raw MS data files were analyzed by Protein Pilot®, version 2.0, using the non-redundant human gene database. Proteins, and their confidences and L/H ratios, were returned with GenInfo Identifier (gi) numbers. Differential regulation within each experimental dataset was determined by Z-score normalization of each dataset, using a confidence of >1.960σ as previously described [12].

2.3. Cell Fractionation

At 5 and 24 h post-infection (hpi), L and H cells were collected and counted. Equivalent numbers of L and H cells were mixed together, mixed cells were washed 3× in >50 volumes of ice-cold Phosphate Buffered Saline (PBS), washed cells were lysed with 0.5% NP-40 supplemented with 1.1 μM peptatin A, incubated on ice for 30 min, and nuclei removed by pelleting at 5000 × g for 10 min. Nuclei were processed by a previously-described high salt/urea double extraction procedure [48] and both fractions frozen at -80°C until further processing. Fractionated samples were probed with antibodies targeting nuclear and cytoplasmic proteins to ensure the method for fractionation was complete (Fig. 5A). Histone H3 is a nuclear protein, Lamin is predominantly found in the nuclear envelope, Actin is typically found in the cytoplasm and tubulin is found in both the nucleus and cytoplasm.

2.4. Mass Spectrometric Sample Preparation and Analysis

Protein content in the various fractions was determined using a BCA™ Protein Assay Kit (Pierce; Rockford, IL) and BSA standards. Samples were then reduced, alkylated and digested with trypsin as previously described [12]. Digested peptides were separated by 2D RP (reversed-phase) high pH – RP low pH peptide fractionation [29,83] and analyzed on a QStar Elite mass spectrometer (Applied Biosystems, Foster City, CA) run in a data-dependent MS/MS acquisition mode as previously described using the manufacturer’s “smart exit” (spectral quality 5) settings [12]. Previously targeted parent ions were excluded from repetitive MS/MS acquisition for 60 x (50 mDa mass tolerance). Protein Pilot 2.0 (Applied Biosystems) software was used for protein identification and quantitation. Raw data files (30 in total for each run) were submitted for simultaneous search using standard SILAC settings for QStar instruments. Proteins for which at least 2 fully trypsin digested L and H peptides were detected at >99% confidence were used for subsequent comparative quantitative analysis. Raw MS data files were analyzed by Protein Pilot®, version 2.0, using the non-redundant human gene database. Proteins, and their confidences and L/H ratios, were returned with GenInfo Identifier (gi) numbers. Differential regulation within each experimental dataset was determined by Z-score normalization of each dataset, using a confidence of >1.960σ as previously described [12].

2.5. Cellular Protein Expression

A549 cells were seeded 24 h prior to use so that they were 80% confluent at the time of infection. Cells were washed with DMEM supplemented with 0.1%BSA and incubated at an MOI of 7 for 1 h. The virus inoculum was removed, cells were washed with PBS, and fresh DMEM supplemented with 0.1% BSA and 0.5 μg/ml TPCK-trypsin was added to the cells. Cells were harvested at 5 h and 24 h post infection. Total cell lysates were collected by washing the cells once with PBS and lysing cells with 2% SDS for immunoblot analysis. Fractionated lysates were harvested by washing the cells with PBS, adding 0.5% NP40 supplemented with Complete Protease Inhibitor (Roche) to the cells and incubating on ice for 30 min. The lysates were then collected and centrifuged at 2500 xg for 10 min to generate soluble cytoplasmic and pelleted nuclear fractions. Each fraction was brought up to equal volumes with a final concentration of 2% SDS for gel electrophoresis and immunoblot analysis. Immunoblots were performed using commercially available primary antibodies coupled with secondary antibodies containing a conjugated IRDye® (Supplementary Table S2). Blots were visualized using a Licor® Odyssey scanner. Band intensities were quantified by densitometry using ImageJ software and normalized to the expression levels of actin. Each experiment was replicated at least 3 times, the means and standard errors are graphically presented. A one-way Anova with a Dunnett post-test was used to determine any significant changes between the various virus strains tested, and a one-way...
Anova with a Bonferroni post-test was used to determine any significant changes between the fractions tested.

2.6. mTOR/AKT Pathway Analysis

A549 cells were seeded 24 h prior to use so that they were 80% confluent at the time of infection. Cells were washed with DMEM supplemented with 0.1% BSA and infected at an MOI of 7 for one hour. The virus inoculum was removed, cells were washed with PBS, and fresh DMEM supplemented with 0.1% BSA and 0.5 μg/ml TPCK-trypsin was added to the cells. Cells were harvested at 5 and 24 hpi. Media were removed and the cells were washed with PBS. Ice-cold 1× MILLIPLEX® MAP Lysis Buffer (EMD Millipore, Merck KGaA, Darmstadt, Germany) was added to the cells. Cells were collected and incubated at 4°C with gentle rocking for 10–15 min. Viruses in samples were inactivated by 5MRADS of gamma irradiation for safe removal from BSL-4.

2.4. mTOR/AKT Pathway Analysis

A549 cells were seeded 24 h prior to use so that they were 80% confluent at the time of infection. Cells were washed with DMEM supplemented with 0.1% BSA and infected at an MOI of 0.1 for 1 h. The virus inoculum was removed, cells were washed with PBS, and fresh DMEM supplemented with 0.1% BSA, 0.5 μg/ml TPCK-trypsin and (0–10 nM) rapamycin was added to the cells. Media were replaced at 5 or 24 hpi with fresh DMEM supplemented with 0.1% BSA and 0.5μg/ml TPCK-trypsin. Infections were allowed to continue for a total of 48 h. Supernatants were harvested and viral titers were quantified by an endpoint Spearman–Kärber TCID₅₀ calculation. Using GraphPad's model comparison analysis, the results were compared to two models, a horizontal line, which indicates that there are no changes in response to rapamycin treatment, and a 4-parameter dose-response curve, where rapamycin has a dose-dependent effect on viral replication. The predicted model and its probability of being correct was reported.

3. Results

3.1. Influenza Virus Infection Induces Significant Up- and Down-Regulation of Numerous Cellular Proteins

A549 cells were infected with the highly pathogenic 1918 influenza virus strain (IVAV1918). Preliminary analyses of cell viability throughout infection indicated that 24 hpi was the optimal time to process the cells for analysis, since signs of infection were present but the majority of cells were still viable (Fig. 1A and B). Furthermore, immunostaining for viral non-structural protein 1 (NS1) confirmed that >80% of cells were infected by this time under our experimental conditions (Fig. 1C). Thus, we selected 5 hpi as an early time point and 24 hpi as a later time point for analyses.

Infected and mock-infected cells were harvested, separated into nuclear and cytoplasmic fractions, and lysates were processed for mass spectrometry analyses. Each experimental analysis identified ~1350–2050 proteins in the cytoplasmic fractions and ~450–950 proteins in the nuclear fractions at ±99% confidence and with ≥2 peptides (Fig. 2A), leading to the overall identification of 3020 proteins from 113,485 L/H peptide pairs. The significance of protein dysregulation was assessed by multiple means. Significance of proteins detected multiple times were determined by t-test. In addition, to facilitate inter-experiment comparisons, and to assess significance of proteins detected only a single time, but with ≥2 non-redundant peptides, all L/H ratios were converted into Z-scores to determine each protein's quantitative deviation from each population's mean as described [12]. Protein dysregulation was considered significant if t-test p values were ≤0.05. Protein dysregulation was also considered significant if the protein was detected and measured multiple times and each of its Z-scores were ≥1.960 or ≤−1.960 (≈95% confidence). Z-scores ≥2.576 or ≤−2.576 (≈99% confidence) were considered significant if proteins were detected and measured only a single time. For further stringency, average fold-change cut-offs of ±75%, which resulted in fold-change ≥1.5-fold, if upwards, or ≤0.667-fold, if downward, compared to mock were applied to proteins detected multiple times and fold-change cut-offs of ±75%, representing ≥1.75-fold if upwards or ≤0.5714-fold if downward compared to mock were applied to proteins detected only a single time. Label swapping identified 24 proteins (including keratin, S100 calcium binding proteins, and albumin pre-protein) that were significantly regulated in one direction under one L/H labeling condition, but significantly regulated in the opposite direction under reciprocal labeling conditions and which thus likely represent contaminants; these were computationally removed from the dataset and from further consideration as described [48]. By using these Z-score criteria and removal of probable contaminants, we identified a total of 79 proteins that were significantly up-regulated at either 5 or 24 hpi in either the cytoplasmic or nuclear fractions and 98 proteins that were significantly down-regulated (Table 1). A few proteins were found significantly regulated in multiple time points or sub-cellular fractions. For example, the 205kD nucleoporin (Nup205) protein was up-regulated in the cytoplasmic fraction at both 5 and 24 hpi, SC11A was up-regulated at 24 hpi in both the cytoplasmic and nuclear fractions, the lamin B receptor (LBR) was up-regulated in nuclear fractions at both 5 and 24 hpi, and the β-induced transforming growth factor (BGFH) was down-regulated at 5 hpi in both the cytoplasm and nucleus (Table 1). A few proteins (H2B1C, SP02B, RFC5, RM04, IBP7, and RAB3B) were consistently up-regulated in one sample but consistently down-regulated at either another time point or in another sub-cellular fraction, suggesting either temporal or spatial re-distribution.

Up-regulated proteins are associated with responses to stress, stimuli and virus, acetylation, cell structure, defense responses, and protein binding, whereas down-regulated proteins are associated with alternative splicing, localization, transport, protein binding, and nucleoside, nucleotide and nucleic acid metabolism.

Proteins, and their levels of regulation, were analyzed by DAVID [17,36,37] and by Ingenuity Pathways Analysis (IPA). These analyses measured numerous classes of proteins (Fig. 3A, left). While many members of most classes of proteins were up or down-regulated at 5 hpi, it is striking that all proteins in the phosphatases, kinases, transmembrane receptors, and translation regulator classes were upregulated, while all cytokines and a predominant fraction of transporters were downregulated (Fig. 3A, middle). By 24 hpi, there was a very evident shift in the expression patterns for members of some classes of proteins. For instance, transcription regulators, which showed mixed up or down regulation at the early time point were exclusively downregulated at 24 hpi. Transmembrane receptors and translation regulators that were all up-regulated at 5 hpi were all down-regulated by 24 hpi, while most kinases, which were all up-regulated at 5 hpi were mostly down-regulated by 24 hpi. The phosphatases were further enriched among the up-regulated proteins by 24 hpi. IPA identified 10 pathways with 5 or more focus molecule members identified from SILAC analysis among the regulated proteins. The top 5 pathways, each with 14 or more identified molecules, were cellular movement,
developmental disorders, cell cycle, molecular transport, and cellular development (Fig. 3B; Supplementary Fig. S1). There were large numbers of proteins whose quantities changed when comparing 5 hpi pathways to 24 hpi pathways. For example, LBR was up-regulated at both 5 and 24 hpi but SMAD2 (and many other pathway members) were unaffected at 5 hpi and significantly down-regulated at 24 hpi (Table 1, Fig. 3B, Network 1). Similarly, most of the other top pathways had larger numbers of down-regulated proteins at the later time point. IPA identified numerous canonical pathways, diseases and biological functions that were significantly dysregulated, and/or that were predicted by Z-score analysis to be highly positively or negatively activated (Table 2). These observations were also complemented by DAVID analyses, which identified various proline dioxygenases and nucleotide binding activities (among others) as major up- and down-regulated molecular functions, and oxygen reduction and macromolecular complex organization and assembly as major biological processes (Fig. 4). The PI3K/mTOR/Akt canonical signaling pathway was one of the significantly affected ones (Table 2; Supplementary Fig. S2), supporting some of our recent observations about the role of this pathway in IAV replication [96].

To validate results obtained by mass spectrometry, we selected five differentially regulated proteins from Table 1, which targeted the various network and canonical pathways. These targeted proteins demonstrated clear changes in regulation, were detected in multiple experiments by mass spectrometry, and had reliable antibodies available for use. Using a similar experimental design as in the SILAC experiments, cells were infected at high multiplicity of infection (MOI) with 1918 virus (1918). The cells were fractionated into cytoplasmic and nuclear components and the expression levels of kinesin family 22 protein (KIF22), LBR, nuclear export factor 1 (NXF1), Nup205 and Rab5b were assessed (Fig. 5). The non-biased SILAC screen demonstrated KIF22 up-regulation in the nuclear fraction at 5 hpi (Table 1) and that the protein was not detected in the cytoplasm or at later time points. Immunoblot analysis confirmed a statistically significant upregulation of KIF22 in the nucleus at 5 hpi but also demonstrated continued elevated expression at 24 hpi. In addition, KIF22 was detected in the cytoplasm although there was no statistically significant change observed with infection (Fig. 5B). The SILAC screen indicated LBR was up-regulated in the nucleus at 5 hpi but also demonstrated continued elevated expression at 24 hpi (Fig. 5C). Although LBR was not detected in the cytoplasmic fractions by SILAC, immunoblot analysis, which is generally more sensitive, showed that LBR was also significantly up-regulated in the cytoplasm by 24 hpi (Fig. 5C). SILAC showed a 5-fold (2.2 log2-fold) decrease in NXF1 abundance in the nuclear fraction at 5 hpi and a no decrease at 24 hpi (Table 1) and immunoblot analysis demonstrated a slight decrease in NXF1 nuclear protein expression at 5 hpi and then a slight up-regulation at 24 hpi (Fig. 5D). While NXF1 was detected in
SILAC cytoplasmic fractions, its expression level was not significantly altered due to infection. However, immunoblot analysis showed it slightly up-regulated at both time points (Fig. 5D). A lower level of NXF1 was expressed in the nucleus than in the cytoplasm, and infection resulted in reduced levels by 5 hpi that rebounded above the mock level by 24 hpi. Cytoplasmic Nup205 expression was similarly elevated when assessed by both SILAC and immunoblotting methods at 5 and 24 hpi (Table 1, Fig. 5E) but remained unchanged in the nucleus. Finally,
### Table 1
A549 cell proteins affected by 1918 infection.

| Accession | HGNC | Name                                      | Count 5 | I : M^2 | Count 24 | I : M^2 | Count 5 | I : M^2 | Count 24 | I : M^2 |
|-----------|------|-------------------------------------------|----------|---------|----------|---------|----------|---------|----------|---------|
| gi|72534670 | PLAP | Phospholipase A2-activating protein     | 2        | 2.98    | 3        | 0.97    | 0        |          | 0        |
| gi|6005860 | RL35 | Ribosomal protein L35                   | 2        | 1.73    | 1        | 1.67    | 2        | 1.19     | 3        | 0.98    |
| gi|56090582 | IR6Q | Immunity-related GTPase family, Q      | 2        | 1.60    | 3        | 0.80    | 0        |          | 0        |
| gi|59850762 | UACA | Uveal autoantigen with coiled-coil domains and ankyrin repeats isoform 1 | 3        | 1.58    | 3        | 0.79    | 0        |          | 0        |
| gi|7706501 | WBP11 | WW domain binding protein 11            | 2        | 1.56    | 0        |          | 0        |          | 0        |
| gi|22538442 | CATZ | Cathepsin Z preproprotein                | 2        | 1.55    | 2        | 1.24    | 0        |          | 0        |
| gi|18087829 | ZC3HAV1 | Zinc finger CCCH-type, antiviral 1-like | 2        | 1.52    | 0        |          | 0        |          | 0        |
| gi|221625487 | IMPA1 | Insolubilizing; (myr)-1 (or 4)- monophosphatase 1, isoform 2 | 3        | 1.00    | 2        | 12.02   | 0        |          | 0        |
| gi|4506019 | 2ABA | Alpha isoform of regulatory subunit 85S, protein phosphatase 2 | 2        | 1.00    | 3        | 4.13    | 0        |          | 0        |
| gi|4506371 | RAB5B | RAB5B, member RAS oncogene family       | 0        |          | 2        | 4.01    | 1        | 0.72     | 0        |
| gi|20149498 | FRIL | Ferritin, light polypeptide              | 1        | 1.34    | 2        | 3.91    | 0        |          | 0        |
| gi|4826852 | ACPM | NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa | 3        | 0.98    | 2        | 2.66    | 3        | 1.09     | 2        | 0.99    |
| gi|57634354 | NUP205 | Nucleoporin 205Ada                | 1        | 2.08    | 2        | 2.64    | 0        |          | 0        |
| gi|156104864 | ACOX3 | Acyl-Coenzyme A oxidase 3 isoform a     | 0        |          | 2        | 2.14    | 0        |          | 0        |
| gi|57222565 | PP2AB | Protein phosphatase 2, catalytic subunit, beta isoform | 0        |          | 2        | 1.97    | 0        |          | 0        |
| gi|4506669 | RLA1 | Ribosomal protein P1 isoform 1         | 3        | 1.03    | 3        | 1.97    | 3        | 1.05     | 3        | 0.99    |
| gi|6857824 | jFT27 | RAB, member of RAS oncogene family-like 4 | 0        |          | 2        | 1.90    | 0        |          | 0        |
| gi|4502989 | COX7A2 | Cytochrome c oxidase subunit VIIa polypeptide 2 (liver) precursor | 0        |          | 2        | 1.85    | 3        | 1.12     | 2        | 1.01    |
| gi|134142337 | MRSP1 | ATP-binding cassette, sub-family C, member 1 isoform 1 | 0        |          | 2        | 1.85    | 1        | 0.87     | 1        | 1.13    |
| gi|7657609 | SCC1A | SEC11-like 1                           | 0        |          | 2        | 1.84    | 0        | 0.73     | 1        | 4.20    |
| gi|7706481 | CKB39 | Calcium binding protein 39             | 1        | 1.00    | 2        | 1.78    | 0        |          | 0        |
| gi|15993475 | TRM6 | tRNA methyltransferase 6                | 0        |          | 2        | 1.53    | 0        |          | 0        |
| gi|66933016 | IMDH2 | Inosine monophosphate dehydrogenase 2   | 3        | 1.05    | 3        | 1.03    | 2        | 3.35     | 2        | 1.11    |
| gi|153792590 | HS90A | Heat shock 90kDa protein 1, alpha isoform 1 | 2        | 1.00    | 3        | 1.06    | 2        | 2.50     | 2        | 2.68    |
| gi|37595752 | LBR | Lamin B receptor                        | 0        |          | 0        |          | 2        | 1.92     | 1        | 2.93    |
| gi|116507237 | GRP78 | Heat shock 70kDa protein 5              | 3        | 1.02    | 3        | 1.18    | 3        | 1.76     | 3        | 0.92    |
| gi|4504271 | H2B1C | Histone cluster 1, H2b                 | 1        | 0.01    | 1        | 0.01    | 2        | 1.70     | 0        |          |
| gi|56676371 | CPSF1 | Cleavage and polyadenylation specific factor 1, 160kDa | 0        |          | 0        |          | 1        | 0.90     | 2        | 13.20   |
| gi|222352111 | MK67I | MX67 interacting nucleolar phosphoprotein | 0        |          | 0        |          | 3        | 0.98     | 2        | 1.84    |
| gi|4758790 | NDUS5 | NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q-reductase) | 0        |          | 1        | 1.12    | 2        | 0.97     | 2        | 1.68    |

**Up-regulated**

Detected more than once

**Detected only once**
| Gene | Description | Score 1 | Score 2 | Score 3 | Score 4 | Score 5 | Score 6 |
|------|-------------|---------|---------|---------|---------|---------|---------|
| STK25 | Serine/threonine kinase 25 | 2.16 | 0 | 0 | 0 | 0 |
| ACA9 | Acyl-CoA dehydrogenase family, member 9 | 2.15 | 3 | 1.15 | 0 | 0 |
| EIF2D | Eukaryotic translation initiation factor 2B, subunit 4 delta isoform 1 | 1.97 | 0 | 0 | 0 | 0 |
| SETD3 | SET domain containing 3 isoform a | 1.92 | 0 | 0 | 0 | 0 |
| IPOS1 | Ran binding protein 11 isoform 2 | 1.89 | 0 | 0 | 0 | 0 |
| SPD2B | SH3 and PX domains 2B | 1.85 | 1 | 0.25 | 0 | 0 |
| U2AF2 | U2 (RNA) small nuclear RNA auxiliary factor 2 isoform a | 1.84 | 0 | 0 | 0 | 0 |
| LRS1M1 | Leucine rich repeat and sterile alpha motif containing 1 | 1.83 | 1 | 0.87 | 0 | 0 |
| MGL1 | Monoglyceride lipase isoform 1 | 1.81 | 0 | 0 | 0 | 0 |
| FDTT | Squalene synthase | 1.80 | 3 | 0.61 | 0 | 0 |
| CTBP1 | C-terminal binding protein 1 isoform 2 | 1.78 | 2 | 0.99 | 0 | 0 |
| PIGR | Polymeric immunoglobulin receptor precursor | 0 | 1 | 100.0 | 0 | 0 |
| CNOT3 | CCR4-NOT transcription complex, subunit 3 | 1.41 | 1 | 100.0 | 0 | 0 |
| STAT | Stat3 | 1 | 1 | 100.0 | 0 | 0 |
| CHD3 | Chromodomain helicase DNA binding protein 3 isoform 3 | 1 | 3.80 | 0 | 0 | 0 |
| PTPRJ | Protein tyrosine phosphatase, receptor type, J isoform 1 precursor | 0 | 1 | 2.60 | 0 | 0 |
| VPS16 | Vacuolar protein sorting 16 isoform 1 | 0 | 1 | 2.48 | 0 | 0 |
| H32 | Histone cluster 2, H3a | 0 | 1 | 2.44 | 1 | 0.94 | 0 | 0.98 |
| G0A4 | Golgi autoantigen, golgin subfamily a, 4 | 0 | 1 | 1.91 | 0 | 0 |
| TSPO | Translocator protein isoform P8R | 0 | 0 | 1 | 61.66 | 1 | 1.34 |
| CSRP1 | Cysteine and glycine-rich protein 1 isoform 1 | 0.97 | 3 | 0.95 | 1 | 5.11 | 0 |
| MOHM | Mitochondrial malate dehydrogenase precursor | 1 | 3.13 | 3 | 1.37 | 1 | 3.80 | 2 | 0.77 |
| ENPL | Heat shock protein 90kDa beta, member 1 | 1.06 | 3 | 1.14 | 1 | 3.70 | 3 | 2.09 |
| PDIA1 | Prolyl 4-hydroxylase, beta subunit precursor | 1.04 | 3 | 1.10 | 1 | 2.90 | 2 | 2.18 |
| KIF22 | Kinesin family member 22 | 0 | 0 | 1 | 2.24 | 0 | 0 |
| F10A | Oxidative stress-associated Src activator | 0.92 | 3 | 0.89 | 1 | 2.06 | 1 | 0.91 |
| DHE3 | Glutamate dehydrogenase 1 | 1.00 | 3 | 1.15 | 1 | 1.97 | 1 | 3.78 |
| RSAD2 | Radical S-adenosyl methionine domain containing 2 | 0 | 0 | 0 | 0 | 15.22 |
| TB5A4 | Tubulin, beta 4 | 0 | 0 | 0 | 0 | 15.22 |
| MX1 | Myxovirus resistance protein | 0 | 1 | 0 | 0 | 15.22 |
| IFIE | Eukaryotic translation initiation factor 4E isoform 1 | 0 | 1 | 0.71 | 0 | 1 | 15.22 |
| G3BP2 | Ras-GTPase activating protein SH3 domain-binding protein 2 isoform a | 1.03 | 3 | 0.86 | 0 | 1 | 15.22 |
| CPMS1 | Calpain, small subunit 1 | 0.93 | 3 | 0.90 | 0 | 1 | 15.22 |
| DESM | Desmin | 0 | 0 | 0 | 0 | 15.22 |
| ANX8A | Annexin A8 | 1.00 | 1 | 1.46 | 0 | 14.42 |
| CAPG | Gelsolin-like capping protein | 0.95 | 3 | 1.11 | 0 | 1 | 9.86 |
| GRWD1 | Glutamate-rich WD repeat containing 1 | 2.00 | 2 | 1.05 | 2 | 0.89 | 1 | 5.96 |
| MLF2 | Myeloid leukemia factor 2 | 0 | 0 | 0 | 0 | 1 | 2.95 |
| RM13 | Mitochondrial ribosomal protein L13 | 0 | 1 | 2 | 0.87 | 1 | 2.48 |
| H2BH1 | Histone cluster 1, H2bh | 0 | 0 | 0 | 0 | 1 | 2.20 |
| IKIP | IKK interacting protein isoform 2 | 0 | 0 | 0 | 2 | 0.90 | 1 | 2.12 |
### Down-regulated

**Detected more than once**

| Gene ID | Gene Name | Description | Log2 Ratio | Fold Change | p-value |
|---------|-----------|-------------|------------|-------------|---------|
| g1149999606 | MOG5 | Mannosyl-oligosaccharide glucosidase isoform 1 | 3 | 0.05 | 1 |
| g14507143 | SNX3 | Sorting nexin 3 isoform a | 2 | 0.06 | 1 |
| g14507131 | RUVF | Small nuclear ribonucleoprotein polypeptide F | 2 | 0.09 | 3 |
| g194578885 | CALU | Calumenin isoform b precursor | 3 | 0.19 | 1 |
| g16677723 | RFC5 | Replication factor C5 isoform 1 | 2 | 0.45 | 1 |
| g127436889 | OFUT1 | Protein O-fucosyltransferase 1 isoform 1 precursor | 2 | 0.47 | 3 |
| g114589951 | RAB1 | DNA directed RNA polymerase II polypeptide E | 3 | 0.53 | 2 |
| g48255937 | CD44 | CD44 antigen isoform 2 precursor | 2 | 0.58 | 3 |
| g34740329 | ROA3 | Heterogeneous nuclear ribonucleoprotein A3 | 2 | 0.58 | 3 |
| g30581135 | SMCA1 | Structural maintenance of chromosomes 1A | 2 | 0.59 | 3 |
| g166795301 | PCYOX | Prenylsteine oxidase 1 | 2 | 0.61 | 3 |
| g58331268 | ERC6L | Excision repair protein ERC6-like | 3 | 0.61 | 0 |
| g21040257 | TRUB1 | TRuB pseudouridine (psi) synthase homolog 1 | 2 | 0.62 | 1 |
| g4507467 | BGHI3 | Transforming growth factor, beta-induced, 68KDa precursor | 2 | 0.64 | 3 |
| g114326552 | MON2 | MON2 homolog | 3 | 0.64 | 3 |
| g148728166 | HTA2 | HIV-1 Tat interactive protein 2, 30KDa isoform a | 2 | 0.65 | 1 |
| g6005721 | ERLN2 | ER lipid raft associated 2 isoform 1 | 3 | 0.60 | 3 |
| g208973246 | DHPR | Quinoid dihydropterdine reductase | 2 | 1.22 | 2 |
| g14757826 | B2M6 | Beta 2-microglobulin precursor | 1 | 1.18 | 2 |
| g4557719 | DNL1 | DNA ligase I | 1 | 1.19 | 2 |
| g21264355 | SMCE1 | SW/SNF-related matrix-associated actin-dependent regulator of chromatin e1 | 0 | 0.34 | 1 |
| g7657671 | UBF1 | Upstream binding transcription factor, RNA polymerase I isoform a | 1 | 0.36 | 2 |
| g4503165 | CUL3 | Cululin 3 | 3 | 0.96 | 3 |
| g63252893 | P4HA2 | Prolip 4 hydroxylase, alpha II subunit isoform 2 precursor | 2 | 0.86 | 3 |
| g5031887 | LLP | LIM domain containing preferred translocation partner in lipoma | 2 | 0.94 | 3 |
| g4758334 | FADS2 | Fatty acid desaturase 2 | 1 | 0.45 | 2 |
| g4557769 | KIME | Mavalonate kinase | 2 | 1.07 | 2 |
| g4504151 | GRN | Granulin precursor | 1 | 1.26 | 2 |
| g4506439 | RBBP7 | Retinoblastoma binding protein 7 | 3 | 0.53 | 3 |
| g5174511 | SMAD2 | Smad- and Mad-related protein 2 isoform 1 | 1 | 0.53 | 2 |
| g116734704 | I2BP2 | Interferon regulatory factor 2 binding protein 2 isoform A | 3 | 1.04 | 2 |
| g77354748 | MTNA | Translation initiation factor eIF-28 subunit alpha/beta/delta-like protein isoform 1 | 2 | 0.73 | 3 |
| g156631005 | PSMD8 | Proteasome 26S non-ATPase subunit 8 | 0 | 2 | 0.57 |
| g118130660 | ZC3HF | Erythropoietin 4 immediate early response | 2 | 0.98 | 2 |
| g4504619 | IBP7 | Insulin-like growth factor binding protein 7 | 2 | 0.81 | 3 |
| g46249388 | SERB | Phoshoerine phosphatase | 3 | 1.12 | 2 |
| g4757718 | ALC1A | Actin-like 6A isoform 1 | 0 | 2 | 0.61 |
| g4503243 | CPS1A | Cytochrome P450, family 51, subfamily A, polypeptide 1 isoform 1 | 0 | 3 | 0.62 |
| Gene ID     | Description                                                                 | Log2 Fold Change | Score   | Total Score |
|------------|------------------------------------------------------------------------------|------------------|---------|-------------|
| g|22547138 | RM04 Mitochondrial ribosomal protein L4 isoform a                           | 0                | 1       | 1.16        |
| g|15487670 | NXF1 Nuclear RNA export factor 1 isoform 1                                  | 1                | 1.12    | 2.00        |
| g|38016127 | RRM34 RNA binding motif protein 34                                            | 0                | 0       | 0.55        |
| g|29789090 | RCC2 Regulator of chromosome condensation 2                                  | 3                | 0.95    | 3.82        |
| g|24430146 | NUP153 Nucleoporin 153KDa                                                    | 0                | 0       | 0.58        |
| g|41406064 | MYH1D Myosin, heavy polypeptide 10, non-muscle                               | 3                | 1.03    | 3.92        |
| g|4885409 | VIGLN High density lipoprotein binding protein                               | 3                | 0.92    | 3.81        |
| g|11543023 | UHRF1 Ubiquitin like with PHD and ring finger domains 1 isoform 1            | 0                | 0       | 0.64        |
| g|7657581 | CMC2 Solute carrier family 25, member 13 isoform 2                           | 0                | 0       | 0.66        |
| g|32454741 | SERPH Serine (or cysteine) proteinase inhibitor, clade H, member 1 precursor | 3                | 0.95    | 3.10        |
| g|7705706 | RS27L Ribosomal protein S27-like                                             | 2                | 1.02    | 3.10        |
| g|62912457 | PSCS Pyrroline-5-carboxylate synthetase isoform 2                            | 3                | 0.80    | 3.06        |

**Detected only once**

| Gene ID     | Description                                                                 | Log2 Fold Change | Score   | Total Score |
|------------|------------------------------------------------------------------------------|------------------|---------|-------------|
| g|10863895 | TYB10 Thymosin, beta 10                                                     | 1                | 0.03    | 1.59        |
| g|149944496| UBXN7 UBX domain containing 7                                                | 1                | 0.03    | 0.58        |
| g|157694494| MBQL1A MYB binding protein 1a isoform 1                                      | 1                | 0.03    | 1.10        |
| g|209969695| ISOC2 Isochorema domain containing 2 isoform 1                               | 1                | 0.03    | 1.10        |
| g|28553970 | H2AJ H2A histone family, member J                                           | 1                | 0.03    | 0.64        |
| g|91208423 | TRIP6 Thyroid receptor-interacting protein 6                                 | 1                | 0.03    | 0.66        |
| g|14149680 | ESY1 Extended synaptotagmin-like protein 1                                   | 1                | 0.33    | 1.14        |
| g|13375746 | CFZ11 Hypothetical protein LOC9624                                           | 1                | 0.39    | 0.78        |
| g|22363935 | CI047 Hypothetical protein LOC25427                                          | 1                | 0.44    | 0.88        |
| g|21361380 | SNF8 EAP30 subunit of E1L complex                                            | 1                | 0.45    | 0.90        |
| g|19923750 | RAB38 Member RAS oncogene family                                            | 1                | 0.45    | 1.60        |
| g|22035672 | TRXR2 Thioredoxin reductase 2 precursor                                      | 1                | 0.49    | 0.98        |
| g|5453898 | PIN1 Protein (peptidyl-prolyl cis/trans isomerase) NF1A-interacting 1        | 0                | 0       | 0.02        |
| g|4504277 | H2B2E Histone cluster 2, H2Be                                                | 0                | 0       | 0.17        |
| g|42716297 | CLU Clusterin isoform 1                                                      | 0                | 0       | 0.21        |
| g|52630440 | FKBP8 FK506-binding protein 8                                                | 0                | 0       | 0.22        |
| g|24234683 | UBP11 Ubiquitin specific peptidase 11                                       | 0                | 0       | 0.24        |
| g|18902712 | PDE12 Phosphodiesterase 12                                                   | 0                | 0       | 0.25        |
| g|15011974 | ARHG2 Rho/rac guanine nucleotide exchange factor 2                           | 0                | 0       | 0.25        |
| g|4502741 | CDK6 Cyclin-dependent kinase 6                                               | 1                | 1.28    | 1.38        |
| g|62865635 | ERG1 Squalene epoxidase                                                      | 0                | 0       | 0.30        |
| g|116534706| I2BP2 Interferon regulatory factor 2 binding protein 2 isoform B             | 0                | 1       | 0.33        |
| g|21624639 | CSNKL1A1 Casein kinase 1 alpha 1-like                                       | 0                | 1       | 0.35        |
| g|21735538 | PDCD4 Programmed cell death 4 isoform 2                                     | 0                | 1       | 0.36        |
| g|7661890 | SNX17 Sorting nexin 17                                                       | 2                | 0.91    | 1.82        |
| g|13489073 | EGLN1 Egl nine homolog 1                                                    | 1                | 0.90    | 1.80        |
| g|34577049 | DYST Dystonin isoform 1E precursor                                           | 0                | 1       | 0.42        |
| g|148612809| WNK1 WNK lysine deficient protein kinase 1                                   | 3                | 1.01    | 1.42        |
| g|65786661 | BTBDB BTB (POZ) domain containing 11 isoform a                              | 3                | 0.93    | 0.42        |
| g|55770850 | CP24A Cytochrome P450 family 24 subfamily A polypeptide 1 isoform 1 precursor | 2                | 1.14    | 0.43        |
SILAC showed Rab5b up-regulation in the cytoplasmic fraction at 24 hpi, although it was not detected at the earlier time point and immunoblot showed significant up-regulation of Rab5b at both 5 and 24 hpi (Fig. 5F). Rab5b was not detected in nuclear fractions by mass spectrometry (Table 1), and was barely detectable by immunoblotting. Thus, while not perfect correlation, there was general consistency between the non-biased SILAC method and the more targeted and more sensitive immunoblotting method. Minor differences in degree or direction of measured regulation might be attributable to inherent differences in sampling (partially degraded proteins would not be measured by immunoblot but their peptides would be detected by MS) or by inherent differences in the sensitivity of each method.

3.2. Differential Host Responses Are Induced by Different IAV Strains

We have previously described host proteomic responses to infection by the lab and mouse-adapted IAV strain A/Puerto Rico/8/34 (H1N1) [12] and we also previously showed that highly pathogenic avian influenza virus strains H5N1 and H7N9 induced more profound alterations than the seasonal or 2009 pandemic IAV H1N1 strains [81]. To extend our findings with the 1918 virus to other influenza strains, we then used immunoblotting to determine whether KIF22, LBR, NXF1, Nup205 and Rab5b were similarly or differentially induced or repressed by representatives of each of the other virus subtypes. As a control, the 1918 virus was used to confirm our previous findings, and we included three additional H1N1 strains: A/Mexico/InDRE4487/2009 (Mx10), a 2009 pandemic H1N1 isolate from a patient in Mexico; A/Canada/ RV733/2007 (RV733), a seasonal strain from 2007 that was isolated from an infected patient in Canada; and A/USSR/90/1977 (U77), a patient isolate collected during the re-emergence of the H1N1 subtype in 1977. We also included two avian-origin viruses associated with highly pathogenic infection in humans: A/Vietnam/1203/2004 (V1203), a H5N1 virus isolated from a patient from Vietnam in 2004; and A/Anhui/1/2013 (Anh2013), a H7N9 virus isolated from a patient in China in 2013. A549 cells were infected with the abovementioned panel of viruses at an MOI of 7. Total cell lysates were collected 24 hpi and analyzed by immunoblotting. The 1918-infected cells demonstrated similar protein expression patterns (Fig. 6) as seen in the previous experiment (Fig. 5). There were a few minor changes in expression levels of the five proteins but that would be expected when comparing fractionated samples (Fig. 5) to total cell lysates (Fig. 6). The human H1N1 strains 1918, Mx10, RV733, and U77; and avian-origin H7N9 (Anh2013) viruses all up-regulated expression of KIF22 to similar levels upon infection. Interestingly, there was statistically significant down-regulation of KIF22 expression when cells were infected with V1203 (Fig. 6A, pb 0.01). There was a statistically significant difference between the LBR expression profiles of 1918-infected cells compared to the other strains that were tested. 1918-infected cells had elevated levels of LBR at 24 hpi, while the other tested strains all showed an inhibition of LBR expression (Fig. 6B, pb 0.01). NXF1 expression was mildly elevated in cells infected with the human H1N1 viruses or the avian Anh2013; however, when infected with V1203 there was a significant inhibition of NXF1 expression at 24 hpi (Fig. 6C, pb 0.001). While there was no statistically significant difference in the expression patterns of Nup205 between cells infected with this panel of viruses, U77 and V1203 tended to trend lower when Nup205 expression was assessed (Fig. 6D). Finally, Rab5b expression profiles had a statistically significant difference between 1918-infected cells and the other strains tested. Infection with 1918 caused the highest increase in Rab5b expression, Mx10, U77, RV733 and Anh2013 caused mild elevation, while

| Protein ID | Description | 5 h | 24 h |
|------------|-------------|-----|-----|
| U2B1V      | Ubiquitin-conjugating enzyme E2 variant 1 isoform d | 1.09 | 0.43 |
| ACSL4      | Acyl-CoA synthetase long-chain family member 4 isoform 1 | 0.98 | 0.48 |
| FAS0A      | XAP-5 protein | 0.87 | 0.49 |
| DNAA3      | Dna (Hsp40) homolog, subfamily A, member 3 isoform 1 | 0.53 | 0.00 |
| NDUS7      | NADH-ubiquinone oxidoreductase Fe-S protein 7 precursor | 0.00 | 0.03 |
| TRY3       | Mesotrypsin isoform 1 preprotease | 0.00 | 0.39 |
| TPM2       | Tropomyosin 2 (beta) isoform 2 | 1.01 | 0.93 |
| TIM23      | Translocase of inner mitochondrial membrane 23 (yeast) homolog | 0.00 | 0.52 |
| CADH2      | Cadherin 2, type 1 preprotease | 0.86 | 1.14 |
| IMM'T      | Inner membrane protein, mitochondrial isoform 2 | 0.00 | 0.02 |
| PSB4       | Proteasome beta 4 subunit | 1.11 | 1.12 |
| NQO1       | NAD(P)H menadione oxidoreductase 1, dioxin-inducible isoform c | 0.00 | 0.20 |
| AK1C1      | Aldo-keto reductase family 1, member C1 | 0.95 | 0.23 |
| FINC       | Fibronection 1 isoform 1 preprotease | 0.00 | 2.31 |
| DHB4       | Hydroxysteroid (17-beta) dehydrogenase 4 | 1.04 | 1.23 |
| CD11B      | Cell division cycle 2-like 1 (PITSLRE proteins) isoform 9 | 0.00 | 0.34 |

Significance assumed if measured ≥2 times and up-regulated ≥1.5-fold, or if down-regulated to ≤0.667-fold compared to mock; or if measured only once and up-regulated ≥1.75-fold, or if down-regulated to ≤0.571-fold compared to mock, as determined by t-test or z-score analysis and as described in reference #19.

1Infected: mock-infected ratio.
2Red highlight indicates significant up-regulation; green indicates significant down-regulation. Proteins arranged top-to-bottom in each half of table from most regulated to least regulated and sorted from Cytoplasm 5 h - Nucleus 24 h.
most strikingly infection with V1203 resulted in a decrease in Rab5b expression (Fig. 6E, \( p < 0.001 \)).

Our lab focuses on identifying changes in the host response caused by influenza virus infections for a variety of different strains to better delineate viral life cycles and features of pathogenesis [12,48,81,96]. To provide more evidence that the changes we observed in protein expression profiles correlated with a pathway response required for influenza infection-propagation, we looked at whether infection altered signaling pathways that are linked to expression of the proteins assessed in this study. Previously, utilizing a lab-adapted A/Puerto Rico/8/34 (H1N1) (PR8) strain, we identified changes in apoptosis and autophagy and the PI3K/Akt/mTOR regulatory pathway was suggested.
Table 2
Most significant canonical pathways, diseases and biological functions.

| Top canonical pathways                        | -Log10 p-value | Activation Z score |
|----------------------------------------------|----------------|--------------------|
| EIF2 signaling                               | 64.2           | 3.6927             |
| Regulation of elf4 and p70S6K signaling      | 34.1           | 0.6882             |
| Mitochondrial dysfunction                    | 28.3           |                    |
| Protein ubiquitination pPathway              | 27.5           |                    |
| mTOR signaling                               | 24             | 0.9285             |
| Oxidative phosphorylation                    | 21.9           |                    |
| Remodeling of epithelial adherens junctions  | 20.3           | -0.6882            |
| tRNA charging                                | 16.1           |                    |
| Integral signaling                           | 14.3           | 0.1280             |
| Actin cytoskeleton signaling                 | 13.3           | -0.7620            |
| Caveolar-mediated endocytosis signaling      | 12             |                    |
| NCF2-mediated oxidative stress response      | 11.9           | -0.3651            |
| 14-3-3-mediated signaling                   | 6.33           | 2.1213             |
| HIPPO signaling                              | 6.29           | -2.0000            |
| Hypoxia signaling in the cardiovascular system| 5.12         | 2.0000             |
| Ephrin B signaling                           | 4.84           | -2.2328            |
| Cell cycle: G2/M DNA damage checkpoint regulation| 4.57         | 2.3094             |
| ERK5 signaling                               | 2.26           | 2.6726             |

| Top diseases & bio functions                 |                |                    |
| Cell death and survival                      | -2.2470        |                    |
| Cancer, cell death and survival, organinal injury and abnormalities, tumor morphology | -2.8300 |                    |
| RNA post-transcriptional modification        | -0.1250        |                    |
| Infectious diseases                          | -2.2170        |                    |
| Cellular growth and proliferation            | -0.3250        |                    |
| Protein synthesis                            | -0.7560        |                    |
| RNA post-transcriptional modification        | -0.3440        |                    |
| Cell cycle                                   | -2.6300        |                    |
| Cell-to-cell signaling and interaction       | -2.2720        |                    |

*As determined by Ingenuity Pathway Analysis®.
† Up-arrows indicate pathway or function positive activation; down-arrows indicate negative activation. Bolded Z-score values indicate significance > 1.96 or < -1.96 sigma.

Discussion

A number of genome-wide RNAi screens, mRNA microarray screens and yeast 2-hybrid assays have defined cellular networks that are required for, or manipulated by, influenza infection. These studies have identified >1400 protein targets that may be worth further analysis [92]. The majority of these studies were performed with less pathogenic influenza virus strains and there was very little overlap between most studies. Because viral infection leads to both qualitative and quantitative effects on host gene expression and function, we have complemented these previous studies by performing quantitative proteomic assessments of influenza infections to further define the effects of influenza virus infection on host functions. Our earlier studies focused on quantitative analyses of host protein responses to the mouse-adapted and attenuated PR8 strain in both cultured A549 [12] and primary bronchotracheal cells [48] and on high-pathogenic avian virus strains in A549 cells [81].

We assessed changes in the host’s global protein response after 1918 influenza virus infection by mass spectrometry. A previous study using microarray technology indicated the presence of a strong inflammatory cytokine/chemokine response but a diminished downstream antiviral response in non-human primates infected with 1918 [47]. Interestingly, using mass spectrometry, we identified a small proportion of cytokines...
involved in antigen processing and MHC presentation that were dysregulated (Fig. 2A; Fig. 3A). In contrast to the previous study, which measured mRNA levels, we observed mildly decreased protein expression levels compared to mock-infected cells. This apparent discrepancy in the antiviral responses may be an artefact of using an in vitro cell culture system versus a fully immunocompetent animal model, or a result of

Fig. 4. Gene ontology analyses of up-regulated and down-regulated proteins. The proteins identified in Table 1 were imported into the DAVID gene ontology suite of programs at the NIAID, gene identifications converted by that program, and ontological functions determined by GOTERM. Only ontological functions whose p values are <0.05 are indicated. □ 5 hpi; ■ 24 hpi.

Fig. 5. Host protein expression profiles of nuclear and cytoplasmic fractions from 1918 infected cells. (A) Cells were fractionated and separated into nuclear and cytoplasmic fractions. Immunoblot analysis was conducted to ensure fractionation was complete. Histone H3 is found in the nucleus, Lamin is located in the nuclear envelope, actin is present in the cytoplasm, and α tubulin is situated in both the nucleus and cytoplasm. (B-F) A549 cells were infected with 1918 at an MOI of 7 and harvested at 5 and 24 h post-infection. Cells were fractionated and analyzed by immunoblot for specific proteins identified from the mass spectrometry analysis. Protein expression was quantified by densitometry and normalized to the expression of actin. The fold increase in protein expression was determined by comparing mock and infected samples. The means and standard error of the means were calculated from three separate protein expression experiments. A one-way Anova with a Bonferroni post-test was used to determine any significant changes between the fractions tested. *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.

C. Ranadheera et al. / EBioMedicine 32 (2018) 142–163
variation between two different technologies. Thus, we investigated a more highly affected pathway.

Our proteomic analysis pointed to the dysregulation of the AKT/mTOR pathway by the 1918 virus. Using a variety of cell-based assays we verified the altered expression states of proteins involved in this pathway and the altered phosphorylation state of the AKT/mTOR pathway with 1918 infection. Furthermore, we then demonstrated that among our panel of different viruses, only the 1918 strain was sensitive to the effects of rapamycin, suggesting that 1918 has a unique way of utilizing the AKT/mTOR pathway to promote viral replication by maintaining mTORC1 activation. This distinct response to the modulation of the AKT/mTOR pathway could be a factor which plays a role in conferring the unique and significant virulence and/or transmissibility of this strain.

The PI3K/AKT/mTOR pathway is known to play a role in viral replication of a number of RNA and DNA viruses by regulating apoptosis, cell
survival and host transcription and translation [8,13]. When PI3K is activated, it triggers AKT phosphorylation, which in turn leads to mTOR activation [5,20,22,31,72,73,77–79,84]. mTOR, a serine/threonine signaling kinase, forms two distinct complexes. The first, mTORC1 consists of mTOR, Raptor, Gbl and DEPTOR and acts as a master growth regulator [44,45]. It controls major anabolic processes by activating protein synthesis, lipogenesis and energy metabolism, and inhibiting autophagy [33,38,44]. The second complex, mTORC2, comprised of mTOR, Rictor, GbL, Sin1, PRR5/Protor-1 and DEPTOR regulates cell proliferation [25,72]. mTORC2 functions as a regulatory kinase of AKT and (4) inhibits mTORC1 activation by sequestering it away from the lysosomal compartment which subsequently affects downstream autophagy regulation. (5) The M2-ion channel of influenza virus has a role in subverting autophagy. (6) Nup205 is a component of the nuclear pore complex and acts as a regulatory gate controlling the size of proteins passing through the nuclear envelope. (7) The viral protein NS1 interacts with NXF1 and inhibits cellular mRNA export from the nucleus. (8) NS1 can also activate PI3K; (9) PI3K, mTOR and AKT activation has been shown to inhibit NXF1-mediated export of cellular mRNAs.

It is widely known that influenza virus infection shuts off host transcription and translation by decreasing production of cellular mRNA and the nucleocyttoplasmic transport of cellular mRNA [27,41–43]. Previously, we demonstrated that viral translation increased in response to the suppression of the mTOR pathway during infection with PR8 but production of progeny virus was impaired [96]. This supports our finding, for the observed temporal depression of the mTOR pathway at early time points. This suppression of the the mTOR pathway is necessary to promote viral translation, a necessary requirement to promote viral genome replication and once ample viral proteins have been synthesized, the pathway returns to a steady state level to allow for the continuation of the viral life cycle and production of viral progeny.

The eIF4F complex, made up of eIF4E, a downstream effector molecule of mTORC1 responsible for binding the cap of cellular mRNAs, the RNA helicase eIF4A, and eIF4G, a scaffolding protein, is necessary for protein translation. However, it was demonstrated that when eIF4E is functionally impaired, influenza translation still occurs [9]. The virus was able to overcome the need for eIF4E by the presence of the viral
polymerase. The viral polymerase remains associated with the cap of the viral mRNA and eIF4A and eIF4G are subsequently recruited to maintain a functional eIF4F complex to initiate viral translation [9]. The inhibition of eIF4E activation is the likely mechanism causing the suppression of host translational activities by preventing the formation of a functional eIF4F complex. Viral translation can still occur, since the
viral polymerase is capable of functioning as eIF4E to allow for the formation of the eIF4F complex. Our results demonstrate the inhibition of mTOR, which prevents eIF4E activation, and supports the observation that influenza virus infection inhibits cellular transcription and translation (Fig. 7). Interestingly, we observed that the inhibition of mTORC1 with rapamycin for prolonged periods does not impair virus replication for most of the viruses we tested, whereas our previous study demonstrated that PR8 replication was impaired with prolonged rapamycin treatment [96]. We observed a similar situation for the 1918 virus. PR8, isolated in 1934 and descended from the 1918 virus, is one of the oldest isolates that is routinely studied. The other strains tested in this study were isolated more recently, from 2007 to 2013. We could be observing an evolutionary adaptation in how the viruses use the PI3K/ AKT/mTOR pathway that could be responsible for these differences.

Nucleocytoplasmic transport of molecules across the nuclear membrane is also important for influenza virus replication. NXF1 is essential for the transport of cellular mRNAs, containing exon-exon junctions, out of the nucleus [15,68]. NXF1 is also essential for influenza virus replication [6,32,40,74] but the role of this pathway has not been well defined. While the export of viral RNP structures out of the nucleus occurs through the interaction between NEP and the CREM1 export pathway [58], the NS1 protein is able to interact with NXF1 and prevent the export of cellular mRNAs [74] (Fig. 7). We observed that influenza infection caused NXF1 to be upregulated at later time points, which could be the host’s attempt to subvert the effects of NS1. Alternatively, the virus may also be able to use the NXF1 export pathway in addition to the CREM1 pathway. Interestingly, the influenza NS1 protein is also capable of binding p85α, the PI3K regulatory subunit, and stimulating PI3K kinase activity [20,22,51,77–79] (Fig. 7). However, the activation of PI3K is limited to IAV while PI3K activation remains unchanged by influenza B viruses [21]. One study linked the PI3K/AKT/ mTOR pathway as a regulator of NXF1-mediated cellular mRNA export from the nucleus [67]. Inhibition of PI3K, AKT and mTOR activation allowed for increased NXF1-mediated export of cellular mRNA from the nucleus and activation of the pathway caused retention of cellular mRNA in the nucleus [67] (Fig. 7). We tested three H1N1 strains, 1918, Mx10 and RV733, and two avian-origin influenza strains, V1203 (H5N1) and Anh2013 (H7N9) that exhibit high virulence in human infections. Interestingly, all three H1N1 strains demonstrated a decrease in mTOR and AKT activation; 1918- and Mx10-mediated mTOR and AKT activation occurred at early time points post infection, while the decrease occurred at later time points for RV733, suggesting reduced upstream activation of PI3K, which was confirmed by the observed inhibition of IR and IGF1R activation. Of note, the two avian influenza viruses caused significant AKT activation, suggesting instead the activation of PI3K for these viruses. Interestingly, IR activation was depressed during infection with the avian strains; however, IGF1R remained at baseline levels suggesting some degree of PI3K activation. Thus, influenza virus-induced signaling appears to be strain specific. Our data demonstrated an initial downregulation of NXF1 in the nuclear fraction and an upregulation in the cytoplasmic fraction at 5 h post infection during 1918 influenza virus infection (Fig. 5), suggesting a shift away from normal steady state equilibrium towards nuclear export of mRNA. By 24 hpi, NXF1 expression returned to steady state levels in the nuclear fraction but remained slightly upregulated in the cytoplasmic fraction (Fig.5). This correlated with the observed inhibition of the AKT/mTOR pathway at 5 hpi and then its return to steady state levels at 24 hpi. Interestingly, when the expression profiles of NXF1 were compared to other viruses at 24 hpi, most viruses responded similarly to the 1918 strain (Fig. 6). The inhibition of the AKT/mTOR pathway and increased shift of NXF1 into the cytoplasm at early time points is consistent with increased NXF1-mediated mRNA nuclear export and may indicate a novel role for this pathway in viral replication. Since expression of NS1 is linked to the retention of cellular mRNA in the nucleus [74,81], this may favor the export of viral mRNA. Furthermore, retention of host mRNA in the nucleus could aid in virus-mediated cap snatching, a unique phenomenon in which the first 10–20 nucleotides of cellular pre-mRNA is removed and used as primer for viral mRNA synthesis. Furthermore, the accumulation of cellular mRNA in the nucleus would subsequently decrease host translation allowing the host machinery to be available for viral replication. Our data show that the inhibition of mTORC1 does not affect viral replication for most viruses, confirming the idea that the host will favor virus replication over cellular transcription/translation. This suggests that the viral-induced inhibition of the PI3K/AKT/mTOR pathway and its downstream effect on NXF1 could play a role in diverting host machinery towards supporting viral functionality. Interestingly, the inhibition of mTORC1 has a unique effect on 1918 replication. We observed inhibition of 1918 replication by the prolonged inhibition of mTORC1, suggesting that a balance between mTOR inhibition for some steps in the viral life cycle and its ability to maintain production of progeny virus must be maintained, a unique finding for this strain of influenza.

LBR and Rab5 were two proteins differentially expressed in 1918-infected cells compared to other strains of influenza virus. LBR, a dual function protein, contains an N-terminal domain found in the nucleoplasm where it interacts with B-lamins, heterochromatin and chromatin binding proteins, aiding in the maintenance of chromatin structure as well as nucleus reformation after mitosis, and a C-terminal domain anchoring LBR to the inner nuclear membrane [18,52,54,68,94,95]. The LBR C-terminal domain has steroid reductase activity [64,71,80], which is important for cholesterol synthesis. The role that LBR plays in maintaining the lamina structure of the nuclear membrane may indirectly regulate transport of viral gene products in and out of the nucleus (Fig. 7). Little is known about the interaction between LBR and the PI3K/AKT/mTOR pathway; however, it was recently discovered that AKT is able to phosphorylate Arginine-Serine residues in the N-terminal region of LBR [90]. The phosphorylation of this protein causes a conformational change, detaching the peripheral heterochromatin from the inner nuclear membrane, causing LBR to become highly mobile, thus fostering the destabilization of the nuclear envelope for mitosis [90]. During this process, the stability of the nuclear pores complexes will be compromised, impairing nuclear transport. The inducible sterol reductase activity of LBR could also have a role in the production of lipids required for viral replication (Fig. 7). A previous study identified LBR as being moderately upregulated when A549 cells were infected with a 2009 pandemic H1N1 isolate by mass spectrometry [16]. Unfortunately, we were not able to demonstrate similar results in our study; however, the isolate (Mx10) used in this study was not employed in mass spectrometry experiments, but was used solely for biological validation, an approach which was not done in the previous study. Therefore, a direct comparison of results is difficult. We show that LBR was upregulated at both 5 h and 24 h after infection with 1918, an observation that was distinctively different from the other strains tested in our study. Our findings suggest a novel role of LBR in the 1918 virus life cycle by accommodating increased nuclear stability, promoting viral nuclear transport, and aiding lipid production involved in virus budding and egress; however, further studies to confirm the role of LBR in the 1918 life cycle are needed.

Rab5 is a GTPase which regulates the endocytic pathway [97], controls homotypic fusion of endosomes [3,30], vesicle movement on microtubules [35] and Rab GTPase conversion [70]. The elevated expression of Rab5 GTPases has been implicated in decreasing insulin-mediated activation of PI3K [51] downstream of mTORC1 activation [7,24,50] (Fig. 7). The decline in mTORC1 activity in circumstances of elevated Rab5 is the result of Rab5 misdirecting mTORC1 away from lysosomal compartments and into swollen vacuolar-like structures, preventing mTORC1 from being phosphorylated by Rheb or Rag [7,50]. We showed Rab5b is upregulated during infection with 1918 influenza virus but not by the other strains assessed (Table 1, Figs. 5 and 6), indicating Rab5 expression may have a unique role in the 1918 virus life cycle. These results are consistent with previous reports that Rab5 expression inhibits the PI3K/AKT/mTOR signaling cascade [7,24,50].
Based on these reports, we may extend the possibility that 1918 infection induces an increase in Rab5 expression as a mechanism to inhibit mTORC1 phosphorylation through its ability to sequester mTORC1 away from sites of PI3K/AKT/mTOR signaling activity [7,24,50]. However, our results also demonstrate a temporal depression of the PI3K/AKT/mTOR pathway by the other strains assessed (Fig. 8). While Rab5 may play a role in the depression of this pathway for 1918, it is not the only factor responsible, since 1918 replication was distinctly sensitivity to the inhibition of mTORC1 phosphorylation by rapamycin treatment (Fig. 9). The distinct expression profile of Rab5 and its sensitivity to mTORC1 inhibition by 1918 infection compared to other influenza strains may be correlated; however, follow up investigations into the role of Rab5 would be needed to fully understand this mechanism of action. mTORC1 is known to negatively regulate autophagy; therefore, a decrease in mTORC1 activity by Rab5 expression will have a positive effect on the induction of autophagy activity [50]. Autophagy is responsible for the degradation of cellular organelles and protein aggregates of long-lived proteins [55]. Macroautophagy is the best understood pathway of autophagy and has roles in both innate and adaptive immune responses [75]. A two-membrane vesicle forms around its target to generate an autophagic vesicle. This vesicle will then fuse with late endosomes and lysosomes for substrate degradation. Many viruses have evolved subversion strategies, which allow them to benefit from host-mediated autophagy [4,23,26,34,46,69,85]. The influenza virus

**Fig. 9.** Increased suppression of mTORC1 activation negatively affects 1918 viral replication. A549 cells were infected with various influenza A viruses at an MOI of 0.01. Cells were treated with varying amounts of rapamycin for 5 or 24 h. At 48 hpi, supernatants were harvested and virus was titrated. A model comparison was performed using horizontal line and 4-parameter variable slope models to fit the data. A horizontal line model suggests there was no effect on virus replication by rapamycin treatment, while a 4-parameter variable slope model predicts that there was a dose-dependent response to treatment.

| Virus     | Preferred Model | Probability of the model being correct | Difference in AICc |
|-----------|-----------------|----------------------------------------|--------------------|
| 1918      | Variable slope  | >99.9% 98.5%                           | 25.4 8.4           |
| Mx10      | Horizontal      | 99.1% 99.8%                            | -9.4 -32.2         |
| RV733     | Horizontal      | 97.2% 99.9%                            | -1.0 -13.3         |
| V1203     | Horizontal      | n/d* n/d*                              | n/d* n/d*          |
| Anh2013   | Horizontal      | n/d* n/d*                              | 70.5% n/d*         |

*The lack of variation between the data points prevented the data from being fitted to the variable slope model so a comparison could not be determined because the data points can only fit a horizontal model.*
M2-ion channel protein plays a role in subverting autophagy by blocking fusion of the autophagosome with the lysosome [4,26] and can further utilize the autophagy machinery as a source of materials needed for virus budding and virion stability [4] (Fig. 7). While the 1918 virus may use Rab5 as an inhibitor of the PI3K/AKT/mTOR pathway, subsequently causing the activation of autophagy, other strains may have evolved alternative strategies to do the same thing.

In conclusion, we found that 1918 infection temporally inhibits the mTOR pathway, while effectively promoting viral replication; however, the robust and prolonged depression of mTOR by ramapycin severely impairs 1918 replication. This suggests that 1918 virus-mediated mTOR activity inhibition is tightly regulated both temporally and by degree to promote 1918 replication. Deviation from this balance of activity would have detrimental effects. Our study suggests that infection of A549 human lung cells with influenza viruses alters host nucleocytoplasmic shuttling of molecules across the nuclear membrane, cellular transcription and translation activities, and host synthesis of lipids through a dysregulation of the PI3K/AKT/mTOR pathway. Among the viruses we compared, we observed a unique expression profile of two host proteins, LBR and Rab5b proteins in 1918 infected cells. The temporal increase in Rab5b expression could play key roles in transient depression of the PI3K/AKT/mTOR pathway. Likewise, the general upregulation of LBR could have a role in increased cholesterol synthesis and its overall increase in post-translational phosphorylation by Akt due to increased expression levels, which could have downstream effects on the stability of the nuclear envelope and subsequently nuclear transport. The findings we have presented in this study complement and bridge some gaps between other reports, together providing a more coherent picture of how influenza viruses hijack the host response and the possible mechanisms required to mediate virus replication. Moreover, our work paves the way for further research on the relationship between 1918 infection and the PI3K/AKT/mTOR signaling pathway and the host mechanisms responsible for facilitating virus replication and in determining levels and severity of pathogenesis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.05.027.

Acknowledgements

The authors thank Kolawole Opanubi for expert technical assistance, Dr. Ming Yang for anti-influenza virus NP monoclonal hybridoma cells, and Dr. Yoshihiro Kawaoka for the influenza virus reverse genetics system.

Funding Sources

This research was supported by grants # PAN-83159 and MOP-106713 from the Canadian Institutes of Health Research (CIHR) to KMC, and by general facility funding from the Public Health Agency of Canada to CR and DK.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

CR, DK and KMC designed the study. KMC prepared the SILAC-labeled cells. DK infected and processed the SILAC-labeled cells for mass spectrometry. CR performed other experimental work related to, and prepared, Figs. 1, 5–9. KMC performed statistical and pathway analyses and prepared Figs. 2–4; S1, S2; and Tables 1, 2. KMC performed statistical and pathway analyses and prepared Figs. 2–4; S1, S2; and Tables 1, 2. All authors wrote and edited the text, and approved the final version. genesis.

References

[1] Ahmed R, Oldstone M, Fauci P. Protective immunity and susceptibility to infectious diseases: lessons from the 1918 influenza pandemic. Nat Immunol 2007;8: 1188–93.
[2] Baas T, Baskin CR, Diamond DL, Garcia-Sastre A, Bielefeldt-Ohmann H, Tumpey TM, et al. Integrated molecular signature of disease: analysis of influenza virus-infected macaques through functional genomics and proteomics. J Virol 2006;80:10813–28.
[3] Barbieri MA, Li GP, Colombo MI, Stahl PD, Rab5, an early acting endosomal Gtase, supports in-vitro endosome fusion without Gtp hydrolysis. J Biol Chem 1994;269: 18720–2.
[4] Beale R, Wise H, Stuart A, Ravenhill RJ, Digard P, Randow F. A LC3-interacting motif in the influenza avirulent M2 protein is required to subvert autophagy and maintain viability. J Biol Chem 2012;287:29013–21.
[5] Betz C, Stracka D, Pesciannotti-Baschong C, Frieden M, Damaurex N, Hall MN. mTOR complex 2-Atg signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology. Proc Natl Acad Sci USA 2013; 110:12526–34.
[6] Brass AL, Huang JC, Benita Y, John SP, Krishnan MN, Feeley EM, et al. The IFIPT proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. Cell 2009;139:1243–54.
[7] Bridges D, Fisher K, Zolov SN, Xiong TT, Inoki K, Weisman LS, et al. Rab5 proteins regulate activation and localization of target of rapamycin complex 1. J Biol Chem 2012; 287:29013–21.
[8] Buchkovich NJ, Yu Y, Zampieri CA, Alwine JC. The TORrid affairs of viruses: effects of mammalian DNA viruses on the PI3K-Akt-mTOR signalling pathway. Nat Rev Microbiol 2008;6:265–75.
[9] Cooray S. The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction pathway and the host mechanisms responsible for facilitating virus replication and bridge some gaps between other reports, together providing a more coherent picture of how influenza viruses hijack the host response and the possible mechanisms required to mediate virus replication. Moreover, our work paves the way for further research on the relationship between 1918 infection and the PI3K/AKT/mTOR signaling pathway and the host mechanisms responsible for facilitating virus replication and in determining levels and severity of pathogenesis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.05.027.

Acknowledgements

The authors thank Kolawole Opanubi for expert technical assistance, Dr. Ming Yang for anti-influenza virus NP monoclonal hybridoma cells, and Dr. Yoshihiro Kawaoka for the influenza virus reverse genetics system.

Funding Sources

This research was supported by grants # PAN-83159 and MOP-106713 from the Canadian Institutes of Health Research (CIHR) to KMC, and by general facility funding from the Public Health Agency of Canada to CR and DK.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

CR, DK and KMC designed the study. KMC prepared the SILAC-labeled cells. DK infected and processed the SILAC-labeled cells for mass spectrometry. CR performed other experimental work related to, and prepared, Figs. 1, 5–9. KMC performed statistical and pathway analyses and prepared Figs. 2–4; S1, S2; and Tables 1, 2. All authors wrote and edited the text, and approved the final version.
Neumann G, Kawaoka Y. Genetic engineering of influenza virus gene expression.

Nesvizhskii AI, Aebersold R. Interpretation of shotgun proteomic data - the protein identification problem.

Katze MG, Decorato D, Krug RM. Cellular messenger-RNA translation is blocked at host genes important for influenza virus replication. Nature 2008;454:890-104.

Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18: 1926–45.

Heaton NS, Randall G. Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe 2010;8:422–32.

Hochwalt-S, Severin A, Ahren C, Habermann B, Runge A, Gillooly D, et al. Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. Cell 2005;121:437–50.

Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.

Klein KA, Jackson WT. Picornavirus subversion of the autophagy pathway. Viruses 2012;4:733–75.

Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, et al. An overlapping protein-coding region in influenza virus segment 3 modulates the host response. J Virol 2007;81:19374–8.

Jacinto E, Lorberg A. TOR regulation of AGC kinases in yeast and mammals. Biochem J 2004;378:129–36.

Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18: 1926–45.

Heaton NS, Randall G. Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe 2010;8:422–32.

Hostal i, Jackson D, Chen YT, Krug RM. Nuclear cytoplasmic transport and RNA-dependent translation of influenza virus messenger-Rnas in late adenovirus-infected cells. Cell 1984;37:843–90.

Katze MG, Denhardt DT, Krug RM. Cellular messenger-Rna translation is blocked at both initiation and elongation after infection by influenza-virus or adenovirus. J Virol 1986;60:1027–39.

Katze MG, Krug RM. Metabolism and expression of Rna polymerase-II transcripts in influenza virus-infected cells. Mol Cell Biol 1984;4:2198–206.

Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Eedjument-Bromage H, et al. MTOR regulates phosphatidylinositol 3-phosphate production via the activation of Rab5. Mol Cell 2002;10:1111–20.

Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Eedjument-Bromage H, et al. MTOR defines a rapamycin-insensitive and rapamycin-dependent independent pathway that regulates the cytokine receptor. J Cell Biol 2004;164:1296–302.

Kim DH, Sarbassov DD, Ali SM, Sabatini DM. Phosphorylation and regulation of mTOR by the rictor-mTOR complex. Science 2005;309:1089–101.

Kleuter T, Das SK, Pillai M, Wilkins M, Aaltonen L, Schmitt CA, et al. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science 1998;279:710–4.

Kobasa D, Jones SM, Shinya K, Kashi JC, Coppis J, Elhara H, et al. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007;447:235–9.

Kroeker EA, Ezzati P, Halayko AJ, Coombs KM. Response of primary human airway epithelial cells to influenza infection – a quantitative proteomic study. J Proteome Res 2012;11:4132–6.

La Gruta NL, Kedzierska K, Stamba J, Doherty P, A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol 2007;85:55–92.

Li L, Kim E, Yuan HX, Inoki K, Goraksha-Hicks P, Schiesler RL, et al. Regulation of mTORC1 by the Rab and Adp GTPases. J Biol Chem 2010;285:19705–11.

Liu P, Bridges DL, Ali SM, Latke RR, Chaur KPV, Eedjument-Bromage H, et al. Gamma delta, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell 2003;11: 855–904.

Klein KA, Jackson WT. Picornavirus subversion of the autophagy pathway. J Virol 2003;77:1549–60.

Kobasa D, Jones SM, Shinya K, Kash JC, Coppis J, Elhara H, et al. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007;447:235–9.

Kroeker EA, Ezzati P, Halayko AJ, Coombs KM. Response of primary human airway epithelial cells to influenza infection – a quantitative proteomic study. J Proteome Res 2012;11:4132–6.

La Gruta NL, Kedzierska K, Stamba J, Doherty P, A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol 2007;85:55–92.

Li L, Kim E, Yuan HX, Inoki K, Goraksha-Hicks P, Schiesler RL, et al. Regulation of mTORC1 by the Rab and Adp GTPases. J Biol Chem 2010;285:19705–11.

Liu P, Bridges DL, Ali SM, Latke RR, Chaur KPV, Eedjument-Bromage H, et al. Gamma delta, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell 2003;11: 855–904.

Maiuri MC, Kroemer G. The inner nuclear-membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.

Makatsori D, Kourmouli N, Polioudaki H, Shultz LD, Mclean K, Theodoropoulos PA, et al. The inner nuclear membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.

Maiuri MC, Kroemer G. The inner nuclear-membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.

Maiuri MC, Kroemer G. The inner nuclear-membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.

Maiuri MC, Kroemer G. The inner nuclear-membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.

Maiuri MC, Kroemer G. The inner nuclear-membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.

Maiuri MC, Kroemer G. The inner nuclear-membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.

Maiuri MC, Kroemer G. The inner nuclear-membrane protein lamin B receptor restricts macroautophagic and proteasomal degradation of host genes crucial for influenza A virus propagation. J Virol 2007;81:1539–50.
[93] Xi QR, Cuesta R, Schneider RJ. Tethering of eIF4G to adenoviral mRNAs by viral 100 k protein drives ribosome shunting. Genes Dev 2004;18:1997–2009.

[94] Ye Q, Worman HJ. Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to Drosophila HP1. J Biol Chem 1996;271:14653–6.

[95] Ye QA, Callebaut I, Pezhman A, Courvalin JC, Worman HJ. Domain-specific interactions of human HP1-type chromodomain proteins and inner nuclear membrane protein LBR. J Biol Chem 1997;272:14983–9.

[96] Yeganeh B, Ghavami S, Rahim MN, Klonisch T, Halayko AJ, Coombs KM. Autophagy activation is required for influenza A virus-induced apoptosis and replication. Biochim Biophys Acta 2018 Feb;1865(2):364–78 (29108912).

[97] Zeigerer A, Gilleron J, Bogorad RL, Marsico G, Nonaka H, Seifert S, et al. Rab5 is necessary for the biogenesis of the endolysosomal system in vivo. Nature 2012;485:465–70.