

Proteomic Analysis of Host Responses in HepG2 Cells during Dengue Virus Infection

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Dengue virus infection remains a public health problem worldwide. However, its pathogenic mechanisms and pathophysiology are still poorly understood. We performed proteomic analysis to evaluate early host responses (as indicated by altered proteins) in human target cells during dengue virus infection. HepG2 cells were infected with dengue virus serotype 2 (DEN-2) at multiplicity of infection (MOI) of 0.1, 0.5, and 1.0. Quantitative analyses of DEN-2 infection and cell death at 12, 24, and 48 h postinfection showed that the MOI of 1.0 with 24 h postinfection duration was the optimal condition to evaluate early host responses, as this condition provided the high %Infection (~80%), while %Cell death (~20%) was comparable to that of the mock-control cells. Proteins derived from the mock-control and DEN-2-infected cells were resolved by 2-D PAGE ($n = 5$ gels for each group) and visualized by SYPRO Ruby stain. Quantitative intensity analysis revealed 17 differentially expressed proteins, which were successfully identified by peptide mass fingerprinting. Most of these altered proteins were the key factors involved in transcription and translation processes. Further functional study on these altered proteins may lead to better understanding of the pathogenic mechanisms and host responses to dengue virus infection, and also to the identification of new therapeutic targets for dengue virus infection.

Keywords: Dengue • Virus • Host responses • Liver • Proteome • Proteomics

Introduction

Dengue virus is an enveloped single plus-stranded RNA virus that belongs to the genus *Flavivirus* (family *Flaviviridae*).¹ To date, dengue virus infection remains a public health problem worldwide and can present with a wide spectrum of clinical manifestations, ranging from mild febrile illness or dengue fever (DF) to devastating dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).² Primary dengue virus infection is mostly associated with the mild form (or DF), which is characterized by biphasic fever, intense headache, myalgia, arthralgia, skin rash, lymphadenopathy, and leucopenia.³ The

severe forms of the disease (DHF and DSS) are usually associated with secondary infection⁴ and are characterized by hemorrhage, thrombocytopenia (reduced number of platelets), and increase in capillary permeability. The DSS patients are suddenly deteriorated from hypovolemic shock, a consequence of significant plasma volume loss.⁵

Although it is generally known that viral, host, and environmental factors contribute to the pathogenesis and progression of DHF/DSS,^{6,7} the interplay between the virus and host target cells remains poorly understood. Monocytes and macrophages have been considered as the primary targets of dengue virus infection.⁸ Recent references have also indicated that the liver is another target organ of dengue virus infection, as evidenced by the detection of viral antigens in hepatocytes and Kupffer cells, and by virus recovery from liver biopsies.^{9–12} In an animal model, mouse liver has been suggested to be a major organ for dengue virus replication.¹³ Hepatomegaly and liver dysfunction have been observed with a higher incidence in DHF patients than in DF patients, suggesting that hepatic abnormality is related to the severity of dengue virus infection.^{14–16}

In the present study, proteomics has been applied to evaluate early host responses (as indicated by altered proteins) in human

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hepatocytes (using HepG2 cell line) during dengue virus infection. HepG2 cells were infected with DEN-2 with varying multiplicity of infection (MOI) values of 0.1, 0.5, and 1.0. Quantitative analyses of DEN-2 infection and cell death at multiple postinfection time-points (12, 24, and 48 h) were performed to define an optimal condition of dengue virus infection for the evaluation of early host responses in the present study. The optimal condition should provide the considerably high percentage of DEN-2 infection with the minimal cell death, as to avoid the effect of cell death on the proteome profile. With the optimal condition, proteins derived from whole cell lysate were resolved by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and visualized by SYPRO Ruby, a fluorescence stain. Quantitative intensity analysis was performed to define differentially expressed proteins in the DEN-2-infected cells compared to the mock-control cells. The altered proteins in the DEN-2-infected cells were then identified by peptide mass fingerprinting. 2-D Western blot analysis was then performed to confirm the proteomic data. Potential roles of these altered proteins as the host responses to dengue virus infection are also discussed.

Materials and Methods

Cultivation of HepG2 Cells. HepG2 cells were grown in the growth medium [minimum essential medium (MEM) (GIBCO; Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate]. Cells were maintained at 37 °C in a humidified incubator with 5% CO₂ atmosphere.

Dengue Virus Production and Stock. Dengue virus serotype 2 (DEN-2), strain 16681, was propagated in C6/36 cell line. Briefly, C6/36 cells were grown in L15 medium (GIBCO), supplemented with 10% FBS, 10% tryptose phosphate broth (TPB), 100 U/mL penicillin, and 100 µg/mL streptomycin, and incubated at 28 °C for 2 days. The confluent C6/36 monolayer was incubated with DEN-2 at the MOI of 0.1 in L15 medium, supplemented with 1% FBS, 10% TPB, 100 U/mL penicillin, and 100 µg/mL streptomycin, at 28 °C for 3 h on a rocker. Subsequently, the culture supernatant was replaced with L15 medium supplemented with 1% FBS, 10% tryptose phosphate broth (TPB), 100 U/mL penicillin, and 100 µg/mL streptomycin, and the cells were further incubated at 28 °C until cytopathic effects were observed. The culture supernatant was then collected by centrifugation at 1500 rpm for 5 min, and the virus titer was determined by focus forming assay¹⁷ in pig fibroblast cells (PS Clone D). The DEN-2 stock was then kept at -70 °C until use.

Infection of HepG2 Cells with Dengue Virus (DEN-2). The HepG2 monolayer was trypsinized with 0.25% trypsin/0.25 mM EDTA in PBS and resuspended in the growth medium. Cells were seeded at appropriate density into each flask and incubated for 24 h. DEN-2 from the stock was then added into the flask with the MOI of 0.1, 0.5, and 1.0, at 37 °C for 2 h. Thereafter, the supernatant was removed, and fresh MEM, supplemented with 2% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate, was added into each flask. The cells were then further incubated at 37 °C in 5% CO₂ incubator for 12, 24, and 48 h. Mock HepG2 cells served as the controls,

which were cultured in parallel to DEN-2-infected cells but without DEN-2 infection.

Detection of DEN-2 Antigen and Quantitative Analysis of DEN-2 Infection. Mock-control and DEN-2-infected HepG2 cells were washed with fresh MEM and fixed with 2% formaldehyde in PBS at room temperature for 1 h. The cells were then washed with PBS and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were incubated with mouse monoclonal antibody (Clone 3H5) specific to the envelope protein of DEN-2 at room temperature for 1 h, washed twice with 0.1% Triton X-100 in PBS, and further incubated with rabbit antimouse immunoglobulin conjugated with FITC (Dako, Glostrup, Denmark) at room temperature for 30 min in the dark. Thereafter, the cells were washed once with PBS, and the percentage of cell infection was measured by flow cytometry using FACScan equipped with CellQuest software (BD Biosciences, Palo Alto, CA).

Quantitative Analysis of Cell Death Using Annexin V/Propidium Iodide Double Labeling. Monolayers of mock-control and DEN-2-infected HepG2 cells were trypsinized with 0.25% trypsin/0.25 mM EDTA in PBS and resuspended in the growth medium. The cells were then collected using low-speed centrifugation at 1500 rpm for 5 min and washed twice with ice-cold PBS. After washing, the cells were labeled with annexin V conjugated with FITC (BD Biosciences) in 300 µL of annexin V buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂·2H₂O; pH 7.4) for 15 min in the dark. Subsequently, propidium iodide (BD Biosciences) at the concentration of 1 µg/mL was added and incubated with cells for 5 min before analysis by flow cytometry using FACScan equipped with CellQuest software (BD Biosciences). The cells fixed with 2% formaldehyde and permeabilized with 0.2% Triton X-100 were run in parallel and served as the positive control, while untreated HepG2 cells served as the negative control.

Protein Extraction. The monolayers of mock-control and DEN-2-infected HepG2 cells were trypsinized with 0.25% trypsin/0.25 mM EDTA in PBS and resuspended in the growth medium. The cells were then collected by low-speed centrifugation at 1500 rpm for 5 min and washed three times with PBS. The cell pellets were resuspended in a lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 120 mM dithiothreitol (DTT), 2% ampholytes (pH 3–10), and 40 mM Tris-HCl at 4 °C for 30 min. The insolubilized materials were then removed using centrifugation at 10 000 rpm for 1 min, and protein concentrations in the supernatants were measured using Bio-Rad Protein Assay (Bio-Rad Laboratories; Hercules, CA) based on the Bradford method.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE). A total of 10 gels derived from 10 individual culture flasks (5 for each group) were analyzed in the present study. Totally, 200 µg of protein derived from each culture flask was premixed with a rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 120 mM DTT, 40 mM Tris-base, 2% (v/v) ampholytes (pH 3–10), and bromophenol blue) to make a final volume of 150 µL per sample. The protein mixture was then rehydrated on immobilized pH gradient (IPG) strip, nonlinear pH 3–10, 7 cm long (Amersham Biosciences; Uppsala, Sweden) at room temperature for 16 h. Subsequently, the first dimensional separation or isoelectric focusing (IEF) was performed using Ettan IPGphor II IEF System (Amersham Biosciences) with a step-and-hold mode until a total of 9083 Vh was achieved. The IPG strip was then equilibrated with equilibration

Buffer I (6 M urea, 130 mM DTT, 112 mM Tris-base, 4% SDS, 30% glycerol, and 0.002% bromophenol blue) for 15 min, followed by another equilibration step in Buffer II (6 M urea, 135 mM iodoacetamide, 112 mM Tris-base, 4% SDS, 30% glycerol, and 0.002% bromophenol blue) for 15 min. Thereafter, the strip was placed onto a 12% polyacrylamide slab gel (8 × 9.5 cm), and the second dimensional separation was performed in SE260 mini-Vertical Electrophoresis Unit (Amersham Biosciences) at 150 V for approximately 2 h. Separated proteins were then visualized with SYPRO Ruby fluorescence dye (Bio-Rad Laboratories), and the 2-D gel image was obtained by Typhoon 9200 laser scanner (Amersham Biosciences).

Matching and Analysis of Protein Spots. Spot matching and analysis of protein spots were performed using Image Master 2D Platinum software (Amersham Biosciences). Parameters used for spot detection were (i) minimal area = 10 pixels; (ii) smooth factor = 2.0; and (iii) saliency = 2.0. A reference gel was created from an artificial gel combining all of the spots presenting in different gels into one image. The reference gel was used for determination of existence and difference of protein expression between gels. Intensity volumes of individual spots were obtained and subjected to statistical analysis. Differentially expressed protein spots were subjected to in-gel tryptic digestion and identification by mass spectrometry.

Statistical Analysis. To define differentially expressed protein spots, unpaired Student's *t* test was performed to compare intensity volumes of corresponding spots between mock-control and DEN-2-infected groups, using SPSS software package for Windows (SPSS, Chicago, IL). The criteria for defining spots with significant differences (either increase or decrease) included (i) *P*-values < 0.05, and (ii) the differentially expressed spots must be consistently present (or absent) in all five gels of each group.

In-Gel Tryptic Digestion. The differentially expressed protein spots were excised from 2-D gels, washed twice with 200 μ L of 50% acetonitrile (ACN)/25 mM NH_4HCO_3 buffer (pH 8.0) at room temperature for 15 min, and then washed once with 200 μ L of 100% ACN. After washing, the solvent was removed and the gel pieces were dried by a SpeedVac concentrator (Savant, Holbrook, NY), and rehydrated with 10 μ L of 1% (w/v) trypsin (Promega, Madison, WI) in 25 mM NH_4HCO_3 . After rehydration, the gel pieces were crushed with siliconized blue stick and incubated at 37 °C for at least 16 h. Peptides were subsequently extracted twice with 50 μ L of 50% ACN/5% trifluoroacetic acid (TFA); the extracted solutions were then combined and dried with the SpeedVac concentrator. The peptide pellets were resuspended with 10 μ L of 0.1% TFA and purified using ZipTip_{C18} (Millipore, Bedford, MA). The peptide solution was drawn up and down in the ZipTip_{C18} 10 times and then washed with 10 μ L of 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were finally eluted with 5 μ L of 75% ACN/0.1% formic acid.

Mass Spectrometric Analysis and Peptide Mass Fingerprinting. The trypsinized samples were premixed 1:1 with the matrix solution containing 5 mg/mL α -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% (v/v) TFA, and 2% (w/v) ammonium citrate and deposited onto the 96-well MALDI target plate. The samples were analyzed by the Q-TOF Ultima MALDI instrument (Micromass, Manchester, U.K.) as described previously.^{18,19} The instrument was externally calibrated to less than 5 ppm accuracy over the mass range of *m/z* 800–3000 using a sodium iodide and PEG 200, 600, 1000, and 2000 mixtures and further adjusted with Glu-

Fibrinopeptide B as the near-point lock mass calibrant during data processing. Peptide mass fingerprinting was performed using both MASCOT (<http://www.matrixscience.com>) and ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) search engines. Proteins were identified based on the assumptions that peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. The NCBI (National Center for Biotechnology Information) protein database was used, and the searches were restricted to mammals. A mass tolerance of 100 ppm was used, and only one missed trypsin cleavage was allowed. The significant hit required at least 4 peptide masses to match with the theoretical masses of the protein banked in the NCBI database.

2-D Western Blotting. 2-D PAGE was performed as described above, but with 100 μ g of total protein for each sample. After the completion of 2-D PAGE, proteins were transferred onto a nitrocellulose membrane, and nonspecific bindings were blocked with 5% skim milk in PBS for 1 h. The membrane was then incubated with mouse monoclonal anti-EF-Tu (Santa Cruz Biotechnology; Santa Cruz, CA; 1:500 in 5% milk in PBS) or mouse monoclonal antihuman vinculin (Chemicon International; Temecula, CA; 1:500 in 5% milk in PBS) at room temperature for 1 h. After washing, the membrane was further incubated with rabbit antimouse IgG conjugated with horseradish peroxidase (1:1000 in 5% milk in PBS) (Dako, Glostrup, Denmark) at room temperature for 1 h. Reactive protein spots were then visualized using SuperSignal West Pico chemiluminescence substrate (Pierce Biotechnology, Inc., Rockford, IL).

Results

DEN-2 Infection in HepG2 Cells. HepG2 cells were infected with DEN-2 with varying MOI values of 0.1, 0.5, and 1.0, and the DEN-2-infected cells were further incubated for 12, 24, and 48 h. We used various MOI and multiple postinfection incubation periods to screen for the optimal condition to evaluate early host responses of HepG2 cells to DEN-2 infection. Mock HepG2 cells served as the controls, as they were cultured in parallel to the DEN-2-infected cells, but without DEN-2 infection. At 12 and 24 h postinfection, the morphology of the DEN-2-infected HepG2 cells was indistinguishable from the mock-control cells, regardless of the MOI used (Figure 1). However, at 48 h postinfection, the DEN-2-infected HepG2 cells showed dramatic morphological changes, also known as cytopathic effects,²⁰ including cell rounding, detachment, and formation of large vacuoles in the cytoplasm. These cytopathic effects in the DEN-2-infected HepG2 cells at 48 h postinfection were more obvious when the MOI was greater (Figure 1).

Confirmation of DEN-2 Infection and Quantitative Analyses of the Infection and Cell Death. The DEN-2 infection was confirmed by cytoplasmic staining of DEN-2 envelope protein using monoclonal antibody specific to the DEN-2 envelope protein (Clone 3H5). The data clearly showed that there was intense cytoplasmic staining in the DEN-2-infected HepG2 cells, whereas the mock controls showed negative result. Flow cytometric analysis was used for quantitative analysis of DEN-2 infection in the DEN-2-infected HepG2 cells. Figure 2A clearly shows that %Infection [(number of the infected cells/total number of all cells) × 100%] of the DEN-2-infected HepG2 cells was greater when the MOI was increased, and was greater when the postinfection incubation period was longer. With the MOI of 1.0, %Infection was 30.75% ± 1.71, 80.03% ± 6.37, and 85.02% ± 2.66% at 12, 24, and 48 h postinfection, respectively

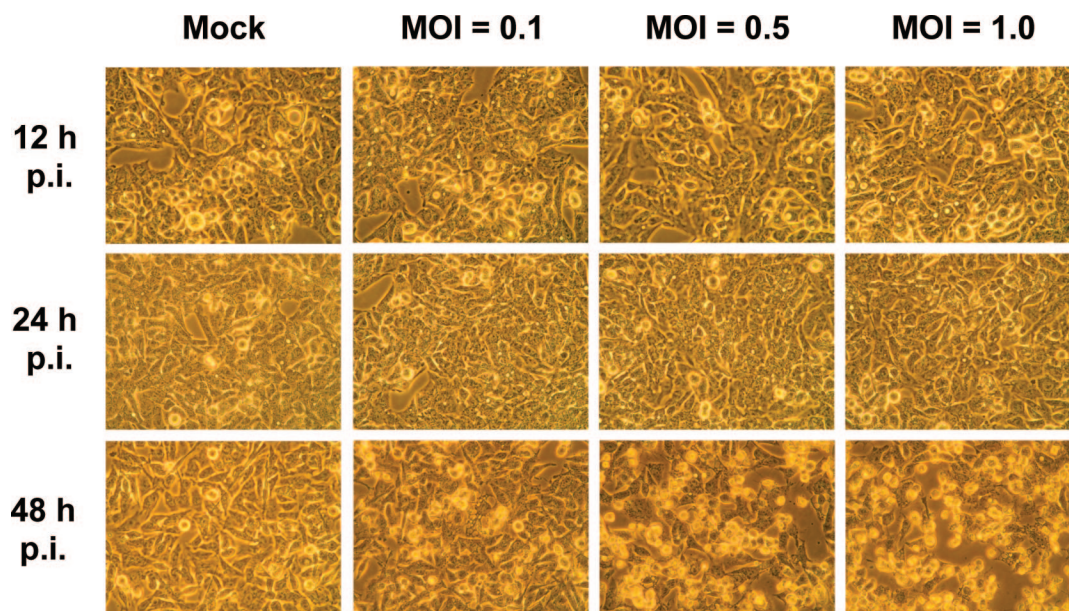


Figure 1. Morphology of the DEN-2-infected HepG2 cells with varying MOI at multiple postinfection time-points. At 12 and 24 h postinfection, the cells infected with DEN-2 with the MOI of 0.1–1.0 had no obvious abnormal morphological findings and looked similar to the mock controls. However, at 48 h postinfection, the infected cells had dramatic detachment of cells and other features of cytopathic effects, which were more obvious when the MOI was greater. Each panel is a representative of one out of three independent experiments. Images were taken with an original magnification of $\times 400$.

($P < 0.05$ for 12 vs 24 or 48 h postinfection, but was not statistically significant for 24 vs 48 h postinfection).

Annexin V/propidium iodide double labeling and flow cytometry were applied to quantitate cell death (both apoptosis and necrosis). Figure 2B shows that %Cell death [(number of the apoptotic and necrotic cells/total number of all cells) $\times 100\%$] at 12 and 24 h postinfection was comparable between the mock-control and DEN-2-infected HepG2 cells, regardless of the MOI used. At 48 h postinfection, the DEN-2-infected HepG2 cells had significantly greater %Cell death compared to the mock-control cells, and %Cell death was greater when the MOI was increased ($27.04\% \pm 6.59$, 43.95 ± 3.37 , and $52.79 \pm 2.55\%$ at the MOI of 0.1, 0.5, and 1.0, respectively).

The objective of the present study was to study early host responses of HepG2 cells to DEN-2 infection as determined by altered proteins; our objective was not to evaluate effects of cell death on the cellular proteome. Therefore, the optimal condition for the present study would be the one that provided the considerably high %Infection, whereas %Cell death was minimal or remained unchanged. Therefore, we selected the MOI of 1.0 and 24 h postinfection time-point as the optimal condition for subsequent proteomic analysis. This condition provided considerably high %Infection ($80.03\% \pm 6.37\%$), while %Cell death ($20.71\% \pm 1.09\%$) was comparable to the mock-control cells (Figures 2).

Altered Proteins in the DEN-2-Infected HepG2 Cells. Alterations in protein expression in the DEN-2-infected HepG2 cells were considered as the early host responses in HepG2 cells to dengue virus infection. With the optimal condition at the MOI of 1.0 and 24 h postinfection incubation duration, the cellular proteome of the DEN-2-infected HepG2 cells was compared to that of the mock controls, using 2-D PAGE approach ($n = 5$ gels derived from 5 individual culture flasks for each group; total $n = 10$ gels). Up to 800 protein spots were visualized in each 2-D gel using SYPRO Ruby staining (Figure 3). Quantitative intensity analysis revealed 17 proteins, of which

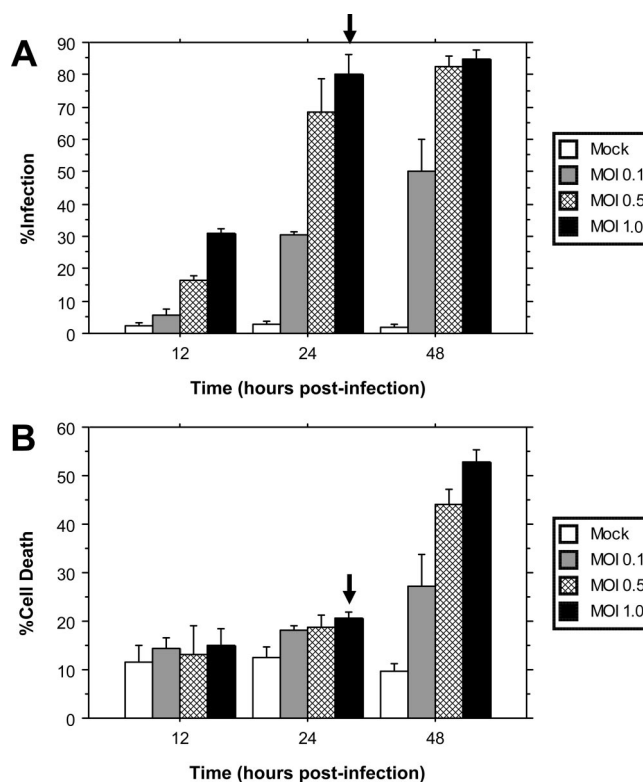


Figure 2. Quantitative analyses of DEN-2 infection and cell death. (A) %Infection [(number of the infected cells/total number of all cells) $\times 100\%$], determined by flow cytometric analysis of cytoplasmic staining of DEN-2 envelope protein. (B) %Cell death [(number of the apoptotic and necrotic cells/total number of all cells) $\times 100\%$], determined by flow cytometric analysis of both apoptotic and necrotic cells using annexin V/propidium iodide double staining. The data are reported as Mean \pm SD of three independent experiments. The arrow indicates the optimal condition selected for subsequent proteomic analysis.

Table 1. Altered Proteins in the DEN2-Infected HepG2 Cells

spot no.	protein	NCBI ID ^a	MOWSE score ^b	Z score ^c	%Cov ^e	p/ MW (kDa)	relative intensity (arbitrary unit)		ratio (DEN-2 / mock)	P
							mock-control (mean ± SEM) ^f	DEN-2-infected (mean ± SEM) ^f		
1	Vinculin	gi131543942	78	2.18	18	5.77/117.22	0.0307 ± 0.0030	0.0068 ± 0.0042	0.22	0.002
2	ERO1-like	gi17657069	88	1.27	41	5.48/55.21	0.0602 ± 0.0108	0.0926 ± 0.0074	1.54	0.038
3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor (Fp) (Flavoprotein subunit of complex II)	gi11169337	158	2.43	46	7.06/73.67	0.1040 ± 0.0105	0.0706 ± 0.0044	0.68	0.019
4	ATP-dependent RNA helicase DDX17 (DEAD box protein 17) (RNA-dependent helicase p72) (DEAD box protein p72)	gi13122595	NA ^d	0.68	19	9.10/72.98	0.2107 ± 0.0801	0.5658 ± 0.0957	2.68	0.022
5	Reticulocalbin 2, EF-hand calcium binding domain	gi14506457	44	1.61	30	4.26/36.91	0.1183 ± 0.0092	0.0722 ± 0.0052	0.61	0.002
6	Calumenin	gi12809324	121	2.43	54	4.47/37.16	0.3845 ± 0.0451	0.2159 ± 0.0303	0.56	0.015
7	Pre-mRNA processing factor 4 homologue (PRP4)	gi156800412	NA ^d	2.43	16	6.70/58.16	0.0000 ± 0.0000	0.0214 ± 0.0088	DIV/0 ^g	0.042
8	Mtmr3 protein	gi120072990	NA ^d	1.34	17	5.50/57.74	0.1559 ± 0.0231	0.0983 ± 0.0077	0.63	0.046
9	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, precursor	gi124660110	95	2.43	41	9.14/60.98	0.2346 ± 0.0334	0.1287 ± 0.0183	0.55	0.024
10	Elongation factor Tu, mitochondrial precursor (EF-Tu) (P43)	gi11706611	103	2.43	38	7.26/49.85	0.0138 ± 0.0021	0.1035 ± 0.0144	7.53	<0.001
11	Retinol dehydrogenase 10	gi125141231	60	NA ^d	26	7.53/38.74	0.0196 ± 0.0026	0.4019 ± 0.0306	20.46	<0.001
12	ATPase, H+ transporting, lysosomal, V0 subunit d1	gi119913432	NA ^d	0.96	50	4.90/40.77	0.1312 ± 0.0070	0.1719 ± 0.0157	1.31	0.045
13	Annexin 5	gi14502107	28	2.43	34	4.94/35.97	0.3126 ± 0.0464	0.5115 ± 0.0067	1.64	0.003
14	Chloride intracellular channel 1	gi14251209	127	2.43	61	5.09/27.25	0.0595 ± 0.0024	0.0785 ± 0.0041	1.32	0.004
15	Dehydrogenase/reductase SDR family member 2 (HEP27 protein) (Protein D)	gi13915733	116	2.43	46	8.90/27.76	1.6826 ± 0.1320	1.1130 ± 0.1893	0.66	0.039
16	Heat-shock protein beta-7 (HspB7) (Heat shock protein 25 kDa 2) (Cardiovascular heat shock protein) (cvHsp)	gi12644226	54	1.24	39	5.84/18.67	0.2240 ± 0.0155	0.3347 ± 0.0383	1.49	0.028
17	Elongin C (Protein p19/6.8)	gi15032161	63	2.18	49	4.74/12.64	0.0000 ± 0.0000	0.5189 ± 0.0528	DIV/0 ^g	<0.001

^a NCBI = National Center for Biotechnology Information. ^b By MASCOT. ^c By ProFound. ^d NA = not available. ^e %Cov = %Coverage of the identified sequence. ^f Data obtained from 5 gels in each group. ^g DIV/0 = divided by zero.

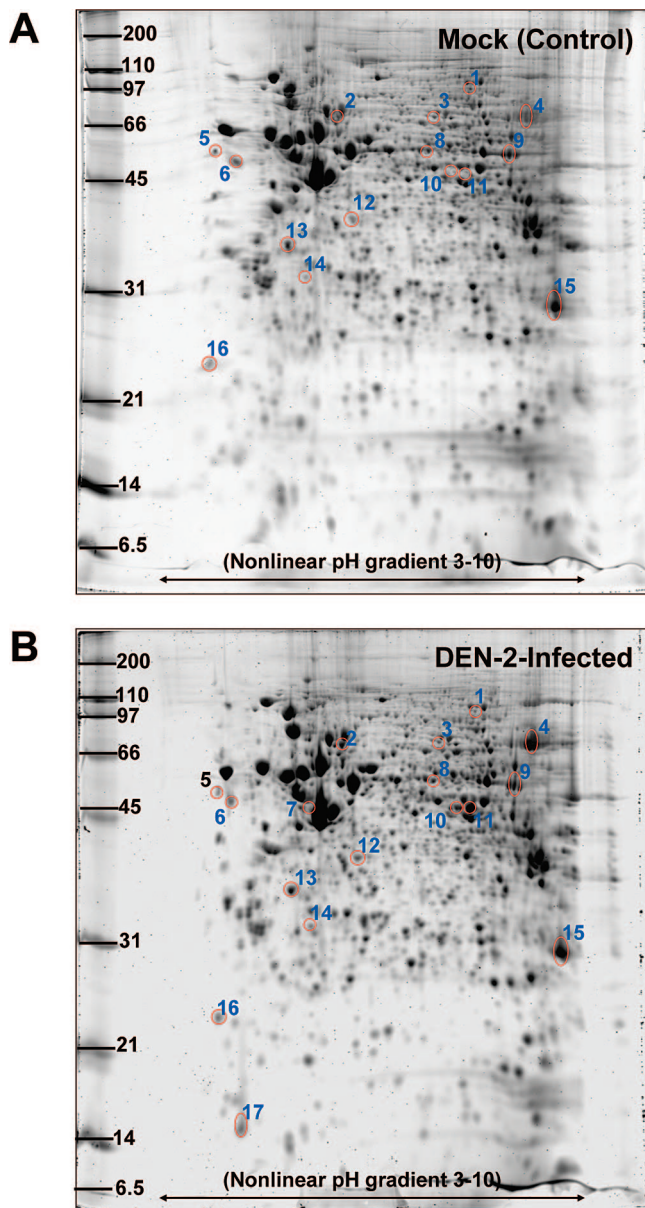


Figure 3. The proteome maps of differentially expressed proteins in mock-control (A) and DEN-2-infected HepG2 cells (B). Each map was created from a representative 2-D gel selected from 5 individual gels in each group. Equal amount of total protein (200 μ g) derived from whole cell lysate of each culture flask was resolved in individual 2-D gel, and the protein spots were visualized by SYPRO Ruby staining. Intensity levels of corresponding spots in all 5 gels of each group were compared to those of the other group. The criteria for defining spots with significant differences (either increase or decrease) included (i) P -values < 0.05 , and (ii) the differentially expressed spots must be consistently present (or absent) in all 5 gels of each group. With these criteria, a total of 17 protein spots (labeled as numbers that correspond to the numbers presented in Table 1) were found significantly differed between the two groups. These differentially expressed proteins were then successfully identified by peptide mass fingerprinting (see Table 1) and classified into various functional groups (see Table 2).

expression levels significantly differed between the two groups (Figure 3). All these 17 differentially expressed protein spots were consistently present (or absent) in all 5 gels of each group and had P -values < 0.05 comparing their means of the intensity

volumes between DEN-2-infected and mock-control samples. All of them were then successfully identified by mass spectrometric analysis and peptide mass fingerprinting (Table 1).

Of these altered proteins, the proteins with increased levels during DEN-2 infection included ERO-1 like; ATP-dependent RNA helicase DDX 17; elongation factor Tu (EF-Tu); retinol dehydrogenase 10; ATPase, H⁺-transporting lysosomal V0 subunit d1; annexin 5; chloride intracellular channel 1; and heat-shock protein beta-7 (HspB7); whereas the proteins of which levels were decreased included vinculin; succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor; reticulocalbin 2; calumenin; Mtmr3 protein; ATP synthase H⁺ transporting, mitochondrial F1 complex, alpha subunit; and dehydrogenase/reductase SDR family member 2 (HEP27 protein). Interestingly, there were two proteins, which were detectable only during DEN-2 infection, including pre-mRNA processing factor 4 homologue (PRP4) and elongin C. These altered proteins were classified into several functional categories based on their functional significance (see Table 2).

Validation of Differentially Expressed Proteins by 2-D Western Blotting. 2-D Western blotting was performed to confirm the proteomic data on two altered proteins; one with increased level and another one with decreased level during DEN-2 infection. Figure 4 clearly confirmed the increase in EF-Tu, whereas Figure 5 clearly illustrates the decreased level of vinculin.

Discussion

There is little information on host responses to dengue virus infection, although there are a few reports recently studied on changes in cellular mRNA expression during dengue virus infection. Because of the lack of an appropriate animal model that can simulate dengue virus infection in humans, *in vitro* experiments have been performed to elucidate dengue virus–host interactions. A study by Liew and Chow²¹ using differential display (DD)-RT-PCR approach allowed characterization of known and novel human genes and expressed sequence tags (ESTs), which were expressed differentially during dengue virus infection in human endothelial-like cells (ECV304). Another study by Warke et al.²² using DD-RT-PCR and microarray approaches demonstrated the activation of several genes with broad functions, including those involved in stress, defense, immune, wounding, inflammatory, and antiviral pathways in human umbilical vein endothelial cells (HUVECs) during dengue virus infection. More recently, Ekkapongpisit et al.²³ reported a number of altered transcripts in the DEN-2-infected HepG2 cells using cDNA-AFLP and semiquantitative RT-PCR.

The altered transcripts reported in the aforementioned studies^{21–23} were not overlapped with the altered proteins found in our present study. These different results are not surprising because changes in the mRNA level do not always correlate with changes in the protein level.²⁴ Additionally, the different types of host cells (ECV304 and HUVEC in the studies by Liew and Chow²¹ and by Warke et al.,²² respectively) should be taken into account for the nonoverlapped data. However, the study by Ekkapongpisit et al.²³ used the same cell line as in our present study (HepG2), but with different conditions of DEN-2 infection. They used the MOI of 5 and most of the differentially expressed transcripts were identified at 72 and 96 h postinfection. In our present study, we carefully selected the optimal condition to define early host responses to dengue virus infection, as to avoid the effect of cell death on the altered cellular proteome because alterations affected solely by early

Table 2. Functional Classification of the Altered Proteins in the DEN2-Infected HepG2 Cells

functional category	down-regulated proteins	up-regulated proteins
1. Protein involved in RNA processing	-	<ul style="list-style-type: none"> • ATP-dependent RNA helicase DDX17 (DEAD box protein p72) • Pre-mRNA processing factor 4 homologue (PRP4)
2. Transcription/Translation factor	-	<ul style="list-style-type: none"> • Elongation factor Tu, mitochondrial precursor (EF-Tu) • Elongin C • Retinol dehydrogenase 10
3. Protein involved in G1 phase cell cycle and cell differentiation	<ul style="list-style-type: none"> • Dehydrogenase/reductase SDR family member 2 (HEP27 protein) 	
4. Metabolic enzyme	<ul style="list-style-type: none"> • ATP synthase, H⁺ transporting, mitochondrial F1 complex, alpha subunit, precursor • Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor (Fp) • Mtmr3 protein (myotubularin related protein 3) 	<ul style="list-style-type: none"> • ATPase, H⁺ transporting, lysosomal, V0 subunit d1
5. Ca²⁺-binding protein	<ul style="list-style-type: none"> • Reticulocalbin 2 • Calumenin 	<ul style="list-style-type: none"> • Annexin 5
6. Structural protein	<ul style="list-style-type: none"> • Vinculin 	-
7. Channel protein	-	<ul style="list-style-type: none"> • Chloride intracellular channel 1
8. Chaperone	-	<ul style="list-style-type: none"> • Heat-shock protein beta-7 (HspB7)
9. Oxidative stress regulatory protein	-	<ul style="list-style-type: none"> • ERO1-like

host responses could easily interfere with the alterations caused by cell death. Nevertheless, the data obtained from analyses of mRNA or protein levels that were altered in different target cells during dengue virus infection at different time-points would be complementary as all of these data are required to fulfill the whole image of both early and late host responses in various target cells during dengue virus infection.

To the best of our knowledge, our present study is the first that addresses changes in the cellular proteome of host cells during dengue virus infection. Unbiased proteomic analysis revealed 17 altered proteins in the DEN-2-infected HepG2 cells (Figure 3 and Table 1). These altered proteins play crucial roles in several cellular functions, particularly in transcription and translation processes (Tables 2). Potential roles of some of these altered proteins in response to dengue virus infection are highlighted as follows.

The translation factors have been well-documented to play crucial roles in viral RNA and protein synthesis.^{25,26} The elongation factors EF-Tu and EF-Ts have been found to bind tightly to the viral RNA-dependent RNA polymerase and have a main function in the delivery of aminoacyl-tRNA (aa-tRNA) to the A site on ribosome during the translation of mRNA to protein.²⁷ In the present study, we observed the increase in EF-Tu level in the DEN-2-infected HepG2 cells. Our finding was consistent with the data reported in a recent study by Jiang et al.²⁸ in which EF-Tu was found to be up-regulated in SARS-CoV-infected cells, as compared to the uninfected cells. Taken together, these data suggest that the increased expression level of EF-Tu may play an important role in the translation of dengue viral RNA.

The elongin complex is a general transcription elongation factor that increases the RNA polymerase II transcription

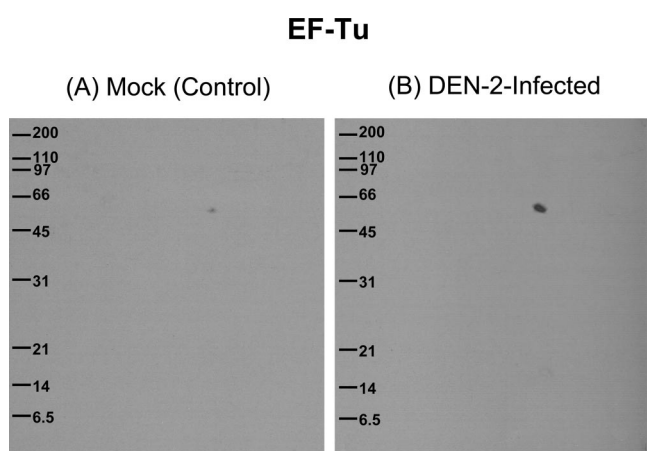


Figure 4. 2-D Western blot analysis of EF-Tu. Equal amount of 100 μ g total protein extracted from each sample was resolved by 2-D PAGE and transferred onto a nitrocellulose membrane. The membrane was then probed with mouse monoclonal anti-EF-Tu antibody and then with rabbit antimouse IgG conjugated with horseradish peroxidase. The immunoreactive protein spot was then visualized using chemiluminescence and autoradiography.

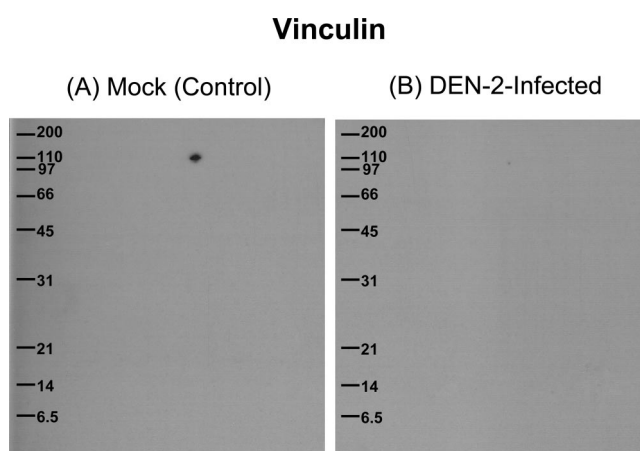


Figure 5. 2-D Western blot analysis of vinculin. Equal amount of 100 μ g total protein extracted from each sample was resolved by 2-D PAGE and transferred onto a nitrocellulose membrane. The membrane was then probed with mouse monoclonal antihuman vinculin antibody and then with rabbit antimouse IgG conjugated with horseradish peroxidase. The immunoreactive protein spot was then visualized using chemiluminescence and autoradiography.

elongation past template-encoded arresting sites.^{29–31} It is a heterotrimer containing three subunits. Subunit A is transcriptionally active and its transcription activity is strongly enhanced by binding to the dimeric complex of subunits B and C (elongin BC complex). The elongin BC complex seems to be involved as an adapter protein in the proteasomal degradation of target proteins via different E3 ubiquitin ligase complexes, including the von Hippel-Lindau (VHL) ubiquitination complex.^{32,33} By binding to BC-box motifs, it seems to link target recruitment subunits, like VHL and members of the SOCS box family, to Cullin/RBX1 modules that activate E2 ubiquitination enzymes.³³

Pre-mRNA processing factor 4 homologue (PRP4) is a member of SR family of splicing factors and belongs to the serine/arginine-rich protein-specific kinase.^{34,35} Phosphorylation of ASF/SF2, an integral part of the spliceosome machinery that is responsible for the regulation of alternative splicing in viral pre-mRNAs, by PRP4 has been shown to affect protein-protein and protein-RNA interactions and to be necessary for splicing.^{35–37} We have postulated that PRP4 may also play an important role in the splicing of DEN pre-mRNA or may be involved in protein-protein and protein-RNA interactions.

Vinculin is a 116-kDa Actin-binding protein that is localized on the cytoplasmic face of integrin-mediated cell-extracellular matrix junctions (focal adhesions) and cadherin-mediated cell-cell junctions.³⁸ Although the major role of vinculin is related to cell structure and integrity, it has been also implicated in modulating cellular signaling pathways that are involved during apoptosis.³⁹ Moreover, there is evidence demonstrating that vinculin-null mouse F9 embryonal carcinoma cells are resistant to apoptosis. The decrease in vinculin in our present study was consistent with the data reported by Go et al.⁴⁰ in which vinculin was clearly shown to be down-regulated in Flock house virus (FHV)-infected cells. Therefore, we have hypothesized that the decrease in vinculin level in the DEN-2-infected HepG2 cells may be involved in inhibition of apoptosis of the host cells and the decrease in apoptotic cell death would be beneficial for dengue virus replication.

Interestingly, PRP4 and elongin C were detectable only in the DEN-2-infected HepG2 cells. These two proteins play crucial roles in mRNA processing and transcription/translation of virus. The presence of these two proteins only in the infected cells might simply implicate their roles in the pathogenic mechanisms of dengue virus infection. Moreover, they may also serve as the new therapeutic targets of dengue virus infection.

In summary, we identified a number of altered proteins in human HepG2 cells during dengue virus infection. Most of these altered proteins were the key factors involved in transcription and translation processes. Further functional study on these altered proteins may lead to better understanding of the pathogenic mechanisms and host responses to dengue virus infection, and also to the identification of new therapeutic targets for dengue virus infection.

Abbreviations: 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; ACN, acetonitrile; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate; CHCA, α -cyano-4-hydroxycinnamic acid; DEN-2, dengue virus serotype 2; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; DTT, dithiothreitol; EF-Tu, elongation factor Tu; FBS, fetal bovine serum; IPG, immobilized pH gradient; MEM, minimum essential medium; MOI, multiplicity of infection; PRP4, pre-mRNA processing factor 4 homologue; TFA, trifluoroacetic acid; TPB, tryptose phosphate broth.

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References

- (1) Chambers, T. J.; Hahn, C. S.; Galler, R.; Rice, C. M. Flavivirus genome organization, expression, and replication. *Annu. Rev. Microbiol.* **1990**, *44*, 649–688.
- (2) Halstead, S. B.; Suaya, J. A.; Shepard, D. S. The burden of dengue infection. *Lancet* **2007**, *369*, 1410–1411.
- (3) Lei, H. Y.; Yeh, T. M.; Liu, H. S.; Lin, Y. S.; Chen, S. H.; Liu, C. C. Immunopathogenesis of dengue virus infection. *J Biomed. Sci* **2001**, *8*, 377–388.
- (4) Hayes, E. B.; Gubler, D. J. Dengue and dengue hemorrhagic fever. *Pediatr. Infect. Dis. J.* **1992**, *11*, 311–317.
- (5) Thein, S.; Aung, M. M.; Shwe, T. N.; Aye, M.; Zaw, A.; Aye, K.; Aye, K. M.; Aaskov, J. Risk factors in dengue shock syndrome. *Am. J Trop. Med. Hyg.* **1997**, *56*, 566–572.
- (6) Halstead, S. B. Pathogenesis of dengue: challenges to molecular biology. *Science* **1988**, *239*, 476–481.
- (7) Monath, T. P. Dengue: the risk to developed and developing countries. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2395–2400.
- (8) Halstead, S. B. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. *Rev. Infect. Dis.* **1989**, *11* (Suppl. 4), S830–S839.
- (9) Kurane, I.; Rothman, A. L.; Livingston, P. G.; Green, S.; Gagnon, S. J.; Janus, J.; Innis, B. L.; Nimmannitya, S.; Nisalak, A.; Ennis, F. A. Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome. *Arch. Virol. Suppl.* **1994**, *9*, 59–64.
- (10) Burke, T. Dengue haemorrhagic fever: a pathological study. *Trans. R. Soc. Trop. Med. Hyg.* **1968**, *62*, 682–692.
- (11) Rosen, L.; Khin, M. M. U T Recovery of virus from the liver of children with fatal dengue: reflections on the pathogenesis of the disease and its possible analogy with that of yellow fever. *Res. Virol.* **1989**, *140*, 351–360.
- (12) Mohan, B.; Patwari, A. K.; Anand, V. K. Hepatic dysfunction in childhood dengue infection. *J. Trop. Pediatr.* **2000**, *46*, 40–43.
- (13) An, J.; Kimura-Kuroda, J.; Hirabayashi, Y.; Yasui, K. Development of a novel mouse model for dengue virus infection. *Virology* **1999**, *263*, 70–77.
- (14) Wahid, S. F.; Sanusi, S.; Zawawi, M. M.; Ali, R. A. A comparison of the pattern of liver involvement in dengue hemorrhagic fever with classic dengue fever. *Southeast Asian J. Trop. Med. Public Health* **2000**, *31*, 259–263.
- (15) Lin, Y. L.; Liu, C. C.; Lei, H. Y.; Yeh, T. M.; Lin, Y. S.; Chen, R. M.; Liu, H. S. Infection of five human liver cell lines by dengue-2 virus. *J. Med. Virol.* **2000**, *60*, 425–431.
- (16) Nguyen, T. L.; Nguyen, T. H.; Tieu, N. T. The impact of dengue haemorrhagic fever on liver function. *Res. Virol.* **1997**, *148*, 273–277.
- (17) Avirutnan, P.; Malasit, P.; Seliger, B.; Bhakdi, S.; Husmann, M. Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. *J. Immunol.* **1998**, *161*, 6338–6346.
- (18) Thongboonkerd, V.; Chutipongtanate, S.; Kanlaya, R.; Songtawee, N.; Sinchaikul, S.; Parichatikanond, P.; Chen, S. T.; Malasit, P. Proteomic identification of alterations in metabolic enzymes and signaling proteins in hypokalemic nephropathy. *Proteomics* **2006**, *6*, 2273–2285.
- (19) Thongboonkerd, V.; Kanlaya, R.; Sinchaikul, S.; Parichatikanond, P.; Chen, S. T.; Malasit, P. Proteomic identification of altered proteins in skeletal muscle during chronic potassium depletion: Implications for hypokalemic myopathy. *J. Proteome. Res.* **2006**, *5*, 3326–3335.
- (20) Marianneau, P.; Steffan, A. M.; Royer, C.; Drouet, M. T.; Kim, A.; Deubel, V. Differing infection patterns of dengue and yellow fever viruses in a human hepatoma cell line. *J. Infect. Dis.* **1998**, *178*, 1270–1278.
- (21) Liew, K. J.; Chow, V. T. Differential display RT-PCR analysis of ECV304 endothelial-like cells infected with dengue virus type 2 reveals messenger RNA expression profiles of multiple human genes involved in known and novel roles. *J. Med. Virol.* **2004**, *72*, 597–609.

- (22) Warke, R. V.; Xhaja, K.; Martin, K. J.; Fournier, M. F.; Shaw, S. K.; Brizuela, N.; de Bosch, N.; Lapointe, D.; Ennis, F. A.; Rothman, A. L.; Bosch, I. Dengue virus induces novel changes in gene expression of human umbilical vein endothelial cells. *J. Virol.* **2003**, *77*, 11822–11832.
- (23) Ekkapongpisit, M.; Wannatung, T.; Susantad, T.; Triwitayakorn, K.; Smith, D. R. cDNA-AFLP analysis of differential gene expression in human hepatoma cells (HepG2) upon dengue virus infection. *J. Med. Virol.* **2007**, *79*, 552–561.
- (24) Klein, J. B.; Thongboonkerd, V. Overview of proteomics. *Contrib. Nephrol.* **2004**, *141*, 1–10.
- (25) Bushnell, M.; Sarnow, P. Hijacking the translation apparatus by RNA viruses. *J. Cell Biol.* **2002**, *158*, 395–399.
- (26) Kushner, D. B.; Lindenbach, B. D.; Grdzlishvili, V. Z.; Noueiry, A. O.; Paul, S. M.; Ahlquist, P. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15764–15769.
- (27) Blumenthal, T.; Carmichael, G. G. RNA replication: function and structure of Qbeta-replicase. *Annu. Rev. Biochem.* **1979**, *48*, 525–548.
- (28) Jiang, X. S.; Tang, L. Y.; Dai, J.; Zhou, H.; Li, S. J.; Xia, Q. C.; Wu, J. R.; Zeng, R. Quantitative analysis of severe acute respiratory syndrome (SARS)-associated coronavirus-infected cells using proteomic approaches: implications for cellular responses to virus infection. *Mol. Cell. Proteomics* **2005**, *4*, 902–913.
- (29) Bradsher, J. N.; Tan, S.; McLaury, H. J.; Conaway, J. W.; Conaway, R. C. RNA polymerase II transcription factor SIII. II. Functional properties and role in RNA chain elongation. *J. Biol. Chem.* **1993**, *268*, 25594–25603.
- (30) Garrett, K. P.; Aso, T.; Bradsher, J. N.; Foundling, S. I.; Lane, W. S.; Conaway, R. C.; Conaway, J. W. Positive regulation of general transcription factor SIII by a tailed ubiquitin homolog. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7172–7176.
- (31) Aso, T.; Lane, W. S.; Conaway, J. W.; Conaway, R. C. Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II. *Science* **1995**, *269*, 1439–1443.
- (32) Duan, D. R.; Pause, A.; Burgess, W. H.; Aso, T.; Chen, D. Y.; Garrett, K. P.; Conaway, R. C.; Conaway, J. W.; Linehan, W. M.; Klausner, R. D. Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science* **1995**, *269*, 1402–1406.
- (33) Kibel, A.; Iliopoulos, O.; DeCaprio, J. A.; Kaelin, W. G., Jr. Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. *Science* **1995**, *269*, 1444–1446.
- (34) Kojima, T.; Zama, T.; Wada, K.; Onogi, H.; Hagiwara, M. Cloning of human PRP4 reveals interaction with Clk1. *J. Biol. Chem.* **2001**, *276*, 32247–32256.
- (35) Gross, T.; Lutzberger, M.; Weigmann, H.; Klingenhoff, A.; Shenoy, S.; Kaufner, N. F. Functional analysis of the fission yeast Prp4 protein kinase involved in pre-mRNA splicing and isolation of a putative mammalian homologue. *Nucleic Acids Res.* **1997**, *25*, 1028–1035.
- (36) Wang, J.; Manley, J. L. Overexpression of the SR proteins ASF/SF2 and SC35 influences alternative splicing in vivo in diverse ways. *RNA* **1995**, *1*, 335–346.
- (37) Xiao, S. H.; Manley, J. L. Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. *Genes Dev.* **1997**, *11*, 334–344.
- (38) Ziegler, W. H.; Liddington, R. C.; Critchley, D. R. The structure and regulation of vinculin. *Trends Cell Biol.* **2006**, *16*, 453–460.
- (39) Subauste, M. C.; Pertz, O.; Adamson, E. D.; Turner, C. E.; Junger, S.; Hahn, K. M. Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. *J. Cell Biol.* **2004**, *165*, 371–381.
- (40) Go, E. P.; Wikoff, W. R.; Shen, Z.; O'Maille, G.; Morita, H.; Conrads, T. P.; Nordstrom, A.; Trauger, S. A.; Uritboonthai, W.; Lucas, D. A.; Chan, K. C.; Veenstra, T. D.; Lewicki, H.; Oldstone, M. B.; Schneemann, A.; Siuzdak, G. Mass spectrometry reveals specific and global molecular transformations during viral infection. *J. Proteome Res.* **2006**, *5*, 2405–2416.

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