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ORIGINAL ARTICLE

Proteomic analysis of differential expression of lung proteins in response to highly pathogenic avian influenza virus infection in chickens

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Abstract

Elucidation of the molecular pathogenesis underlying virus-host interactions is important for the development of new diagnostic and therapeutic strategies against highly pathogenic avian influenza (HPAI) virus infection in chickens. However, the pathogenesis of HPAI virus in chickens is not completely understood. To identify the intracellular signaling pathways and critical host proteins associated with influenza pathogenesis, we analyzed the lung proteome of a chicken infected with HPAI H5N1 virus (A/duck/India/02CA10/2011/Agartala). Mass spectrometry data sets were searched against the chicken UniProt reference database. At the local false discovery rate level of 5%, a total of 3313 proteins with the presence of at least one unique peptide were identified in the chicken lung proteome datasets. Differential expression analysis of these proteins showed that 247 and 1754 proteins were downregulated at 12 h and 48 h postinfection, respectively. We observed expression of proteins of the predominant signaling pathways, including Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs), NOD-like receptors (NLRs), and JAK-STAT signaling. Activation of these pathways is associated with the cytokine storm effect and thus may be the cause of the severity of HPAI H5N1 infection in chickens. We also observed the expression of myeloid differentiation primary response protein (MyD88), inhibitor of nuclear factor kappa B kinase subunit beta (IKBKB), interleukin 1 receptor associated kinase 4 (IRAK4), RELA proto-oncogene NF-κB subunit (RELA), and mitochondrial antiviral signaling protein (MAVS), which are involved in critical signaling pathways, as well as other, less-commonly identified proteins such as hepatocyte nuclear factor 4 alpha (HNF4A), ELAV-like RNA binding protein 1 (ELAVL1), fibronectin 1 (FN1), COP9 signalosome subunit 5 (COPS5), cullin 1 (CUL1), breast cancer type 1 susceptibility protein (BRCA1), and the FYN proto-oncogene Src family tyrosine kinase (FYN) as main hub proteins that might play important roles in influenza pathogenesis in chickens. In summary, we identified the signaling pathways and the proteomic determinants associated with disease pathogenesis in chickens infected with HPAI H5N1 virus.

Introduction

To establish control measures against any emerging or re-emerging infection, it is necessary to gain an adequate understanding of virus-host interactions, which provides essential clues for developing new diagnostic and therapeutic strategies [1]. The high-throughput functional genomics approach provides a deeper understanding of the emerging disease by encompassing both the pathogen and the host response. Virus-host interactions are multidimensional in nature, and these interactions alter various host components such as the transcriptome, the proteome, miRNA, the metabolome, and the lipidome [2]. In past years, most high-throughput studies on H5N1-host interactions have focused on the transcriptome [3].

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However, transcriptomic studies do not provide information about posttranscriptional regulation, posttranslational modifications, or protein-protein interactions [3]. The virus-host interaction involves hundreds to thousands of host proteins. Hence, the application of a high-throughput proteomics approach for the analysis of virus-induced innate immune responses in combination with transcriptomic analysis promises a better understanding of the molecular mechanisms involved in influenza pathogenesis [4]. Proteomics refers to the large-scale study of protein expression, protein-protein interactions, or posttranslational modifications based on high-resolution mass spectrometry [5, 6].

Proteomics studies of influenza virus infections in macaques [7], mice [8], continuous cell lines [9–11], chickens [12, 13], dogs [14], and primary human cells [15–18] have provided information on changes in the host proteome at both the cellular and whole-organism levels. Recent studies of the chicken proteome response to H5N1 infection revealed several alterations in cytoskeleton, metabolic process, cellular component, and transcription regulation proteins [12, 13]. However, detailed information about the signaling pathways and proteomic determinants involved in HPAI H5N1 viral pathogenesis in avian species is not available. In this study, we identified signaling pathways and the proteomic determinants associated with disease pathogenesis in chickens infected with highly pathogenic avian influenza (HPAI) H5N1 virus (A/duck/India/02CA10/2011/Agartala) at the proteome level. The A/duck/India/02CA10/2011/Agartala virus is an HPAI H5N1 virus belonging to H5 clade 2.3.2.1. The virus was isolated from a dead domestic duck during an AIV outbreak in which 60% mortality was observed at the State Duck Breeding Farm of Tripura, India, in 2011. This virus is one of the earliest clade 2.3.2 HPAIV isolates identified in India, and its pathogenic characterization in chickens is therefore important. This virus causes high mortality in ducks and is invariably lethal to domestic chickens.

Materials and methods

Experimental infection of chickens

Six-week-old healthy domestic chickens that were seronegative for avian influenza virus (AIV) were used in this study. They were obtained from the specific-pathogen-free hatchery unit of ICAR-National Institute of High-Security Animal Diseases, Bhopal, India. The animal experiments were approved by the Institutional Animal Ethics Committee of ICAR-NIHSAD (approval no. 68/IAEC/HSADL/12, dated 11.05.2012), and all experiments were conducted in the biosafety level 3 containment facility of ICAR-National Institute of High-Security Animal Diseases, Bhopal, India. Chickens were separated into four groups (5 birds per group). Three of the four groups were inoculated intranasally with 10^6 times the mean embryo infectious dose (EID_{50}) of H5N1 virus (A/duck/India/02CA10/2011/Agartala), and one group (control) was inoculated with phosphate-buffered saline (PBS). The birds were observed daily for clinical signs, and all birds were euthanized by cervical dislocation. The caudal part of the lung tissues was collected from five birds from each infected group at 12, 24, and 48 h postinfection. Lung tissues were collected from the control group at 12 h post-inoculation. The tissues were snap-frozen in liquid nitrogen and stored at -80°C until protein extraction. Avian influenza virus infection of lung tissues was confirmed by virus isolation in embryonated chicken eggs and by RT-PCR.

Protein extraction

150 mg of lung tissue from each sample was washed in 50 mM NH_4HCO_3 washing buffer. The lung tissue was cut into small pieces, and 650 µl of SDS protein extraction lysis buffer (0.1% sodium dodecyl sulfate [SDS, Invitrogen], 50 mM NH_4HCO_3 [Sigma], and 1X cOmplete™ Protease Inhibitor Cocktail [Roche-11836145001]) was added. Tissue samples were homogenized in LZ-Lyser homogenizer at 30 HZ for 2 min. After complete homogenization, the total cell lysate was incubated on ice for 90 min for complete protein extraction. The total cell lysate was centrifuged at 20,000 *g* for 60 min at 4°C, and the supernatant was collected. The supernatants were immediately treated at 56°C for 30 min in a dry bath to inactivate HPAIV H5N1 in the protein extracts. The samples were then stored at -80°C for mass spectrometry analysis.

Sample preparation for LC-MS analysis

The quality of the lung protein preparation was evaluated by 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE). A pool for each time point was prepared by combining 50 µg of protein from each of the three best samples at that time point. The disulfide bonds between sulfhydryl groups of cysteine side chains in the protein samples were reduced by treatment for 1 h at 95°C in 100 mM dithiothreitol, followed by alkylation with 55 mM iodoacetamide for 45 min in the dark at room temperature. Trypsin was added to all protein samples at a 1:20 (wt/wt) trypsin-to-protein ratio, and the samples were incubated overnight at 37°C. After trypsin digestion, the sample quality was again checked by SDS-PAGE. Digested peptide samples were concentrated to a total volume of 50 µl using a vacuum centrifuge. Peptides from uninfected and infected samples were labeled with iTRAQ 4-Plex (P/N: 4352135) reagents. The labels used for sample pools of different groups were as follows: iTRAQ label 114- control; iTRAQ label 115-12hr; iTRAQ label 116-24hr; iTRAQ

label 117-48hr. These iTRAQ-labeled samples were pooled and then purified using strong cation exchange (SCX) chromatography. The fractions from SCX chromatography were collected and pooled into 15 fractions based on retention times (minutes). The pooled fractions were vacuum dried and dissolved in 10 µL of 0.1% formic acid. One microliter of the sample was injected onto a C18 Nano-LC column for separation of peptides, followed by mass spectrometry analysis using a Waters Q-TOF instrument.

Bioinformatics analysis

The Waters-specific raw data set files were converted to proteomics standard mzXML format using the ProteoWizard tool MSconvert with default parameters [19]. A database search and other downstream bioinformatic analyses were done in Trans-Proteomic Pipeline (TPP) [20]. TPP includes modules for validation of database search results, quantitation of isotopically labeled samples, and validation of protein identification. MS/MS ion spectra were searched against the chicken UniProt reference database using the Comet MS/MS sequence database search engine [21]. Data for all 17,719 proteins that comprise the chicken UniProt reference database and 11 protein sequences of HPAIV H5N1 virus (A/duck/India/02CA10/2011/Agartala) from the NCBI protein database were downloaded. The following parameters were used for the database search: precursor/peptide mass tolerance, 1.8 Da; fragment tolerance, 1.6 Da; fixed modification, carbamidomethylation for Cys (C) and iTRAQ N-terminus; variable modification, oxidation (M) and phosphorylation of (STY); and the number of missed cleavages, 2. The peptide-spectrum assignments of the Comet search engine were validated using the PeptideProphet and iProphet tools of TPP [22]. Protein identifications were validated with the ProteinProphet tool of TPP based on PeptideProphet or iProphet results [23]. Proteins identified from the ProteinProphet result were filtered based on protein probability above 0.95 (local false discovery rate level, 5%), and contained at least one unique peptide. This filtered protein list was used for further downstream functional analysis. Up- and downregulation of a particular protein was calculated as infected sample intensity divided by control sample intensity (i.e., 115-12hr/114-control). Likewise, for all proteins and specific postinfection time intervals, fold change values were calculated. Functional classification of the proteins was performed for Gene Ontology (GO) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [24] and for pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/). A heat map was generated using the Clustvis web tool [25]. We used the online web server NetworkAnalyst for the construction of protein-protein interaction (PPI) networks [26]. The main driving or hub proteins were identified based on two topological measures: degree centrality and betweenness centrality.

Meta-analysis of transcriptome datasets

For meta-analysis of chicken transcriptome datasets, we selected microarray datasets from two independent studies. Hu et al. (2015) studied the immune response of primary chicken lung cells infected with two HPAI H5N1 viruses using microarray technology [27]. Ranaware et al. (2016) studied the global immune response of chickens infected with HPAI H5N1 (A/duck/India/02CA10/2011) virus [28]. The microarray datasets were analyzed using the GEO2R online tool of the NCBI. The original submitter-supplied processed microarray data tables were identified using a GEO query. Then, we identified the control and test group samples, and samples belonging to each group were assigned, and the logFC, *p*-value, and adjusted *p*-value were calculated using the Limma R package. Adjusted *p*-values below 0.05 were used as the threshold to find differentially expressed genes.

Bayesian networks were reconstructed for Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs), interleukin 1 receptor type 1 (IL1R), NOD-like receptors (NLRs), and JAK-STAT signaling pathways using the 'bnlearn' package [29]. The networks were constructed based on the intensity values of a microarray dataset from our previously published work [28]. The Bayesian network structure was learned from the transcriptomic dataset with prior knowledge using the hill-climbing (HC) algorithm. After learning the network structure, the conditional probability tables (CPTs) at each node were found by running the bn.fit function, which runs the EM algorithm to learn CPT for different nodes in the learned graph.

Results and discussion

Virus-host interactions are multidimensional, including alterations in the transcriptome, proteome, metabolome, and lipidome of the host. In recent years, most high-throughput studies have focused primarily on the host transcriptomic responses, and other host components have received less attention. This study presents a comprehensive identification of the lung proteome, critical signaling pathways, and proteomic determinants responsible for disease pathogenesis at the proteome level in chicken lung tissues infected with the HPAI H5N1 virus at different time points postinfection.

Clinical signs

Chickens in the control group did not show any clinical signs during the experimental period. In the test group, the birds were normal up to 12 h postinfection. Mild clinical signs such as depression, decreased feed and water consumption, and ruffled feathers were observed at 24 h postinfection. Clinical signs including dullness, lacrimation, cyanotic combs and wattles, edema, and red discoloration of the shanks and feet were seen in the birds at 48 h postinfection.

Raw mass spectra dataset analysis

In total, 15 fractions of Waters QTOF raw data sets were generated, with a data size of 48 GB, including 147,451 MS scans and 19,917 MS/MS scans. A total of 19,917 MS/MS spectra were searched against the chicken protein database using the Comet search engine. The iProphet algorithm identified 17,273 unique peptides and 8,516 unique proteins at a minimum probability threshold of 0.05 and a minimum of 7 amino acids. To increase the accuracy of validation of peptides and proteins, we applied the local false discovery rate level of 5% (probability cutoff, 0.95) in the ProteinProphet output. At a higher probability threshold cutoff, we identified a total of 3313 proteins with the presence of at least one unique peptide. ProteinProphet-predicted sensitivity and error rate information is shown in Supplementary Fig. S1. This is the largest number of proteins identified to date in chickens. Our proteomic approach identified H5N1 viral peptides such as NA, NP, and PB1 in the chicken lung proteome. NA is a sialidase responsible for releasing sialic acid from glycoproteins and glycolipid sialoconjugates of bound influenza virus to assist virus release [30]. NP is an important viral protein responsible for the packaging of the viral RNA and has also been shown to be involved in many aspects of influenza viral replication [31, 32]. Viral protein PB1 is associated with the high pathogenicity of H5N1 viruses in ducks [33]. The influenza virus infection status in lung tissues was confirmed by virus isolation in embryonated eggs and by RT-PCR in addition to identification of viral peptides in the lung proteome dataset.

Differential protein expression analysis

The differentially expressed proteins were identified at 12, 24, and 48 h postinfection. A total of 820, 827, and 693 proteins were upregulated at 12, 24, and 48 h postinfection, respectively. Further, 2493, 2441, and 2620 proteins were downregulated at 12, 24, and 48 h postinfection, respectively, in the chicken lung proteome (Table 1). A total of 470 proteins were found to be upregulated and 2235 were downregulated at all time intervals postinfection in chicken lungs infected with the H5N1 virus (Fig. 1). The protein profile showed that 70, 70, and 101 proteins were upregulated in chicken lung tissue only at 12, 24, and 48 h postinfection, respectively (Fig. 1a). Downregulation of 87, 35, and 245 proteins was observed only at 12, 24, and 48 h postinfection, respectively (Fig. 1b). The fold change in the level of upregulated proteins ranged from 42 to 1. Interestingly, at the 48-h time point (fold change value below 1.5), the number of proteins that were downregulated (n = 1754) was higher than at 12 h postinfection (n = 247) (Table 1). A heat map of differentially expressed proteins in HPAIV-infected lung tissues is shown in Figure 2. The results show that most of the host proteins were downregulated at the later stage of infection.

Table 1 Differential protein expression in chickens infected with HPAI H5N1 virus

Time postinfection	No. of upregulated proteins	No. of downregulated proteins	No. of upregulated proteins (>1.5 fold)	No. of downregulated proteins (<1.5 fold)
12 h	820	2493	138	247
24 h	872	2441	157	222
48 h	693	2620	173	1754

Gene Ontology analysis of the commonly upregulated and downregulated lung tissue proteins of H5N1-infected chicken lung showed enrichment in gene expression related to the cytoskeleton, regulation of the cell cycle, and regulation of protein kinase activity (Table 2). Both upregulation (KRT6A, MCPH1, MYH7, MICAL1, and MICAL3) and downregulation (ACTL9, CTNNB1, DNM1, FILIP1L, and MYO1B) of cytoskeletal proteins were observed in the infected lung tissue. Cytoskeletal proteins have been reported to interact with viral proteins to regulate viral replication and assembly as well as the transport of viral components in the cell [34, 35]. Similar associations of cytoskeletal proteins with influenza virus infection have been reported previously [10, 14, 36]. The cyclin-dependent kinases (CDKs) such as CDK13, damage-specific DNA binding protein 1 (DDB1), dopamine receptor D3 (DRD3), forkhead box G1 (FOXG1), and transcription factor 3 (TCF3), which are involved in the regulation of the cell cycle, were all upregulated in the chicken lung proteome. Söderholm *et al*. reported that cyclin-dependent kinase activity is required for efficient viral replication and activation of the host antiviral responses [37]. Proteins associated with protein kinase activity, namely, Janus kinase 3 (JAK3), microtubule affinity regulating kinase 3 (MARK3), TANK binding kinase 1 (TBK1), eukaryotic translation initiation factor 2 alpha kinase 3 (EIF2AK3), protein kinase C alpha (PRKCA), and transient receptor potential cation channel subfamily M member 6 (TRPM6) were downregulated in the chicken lung

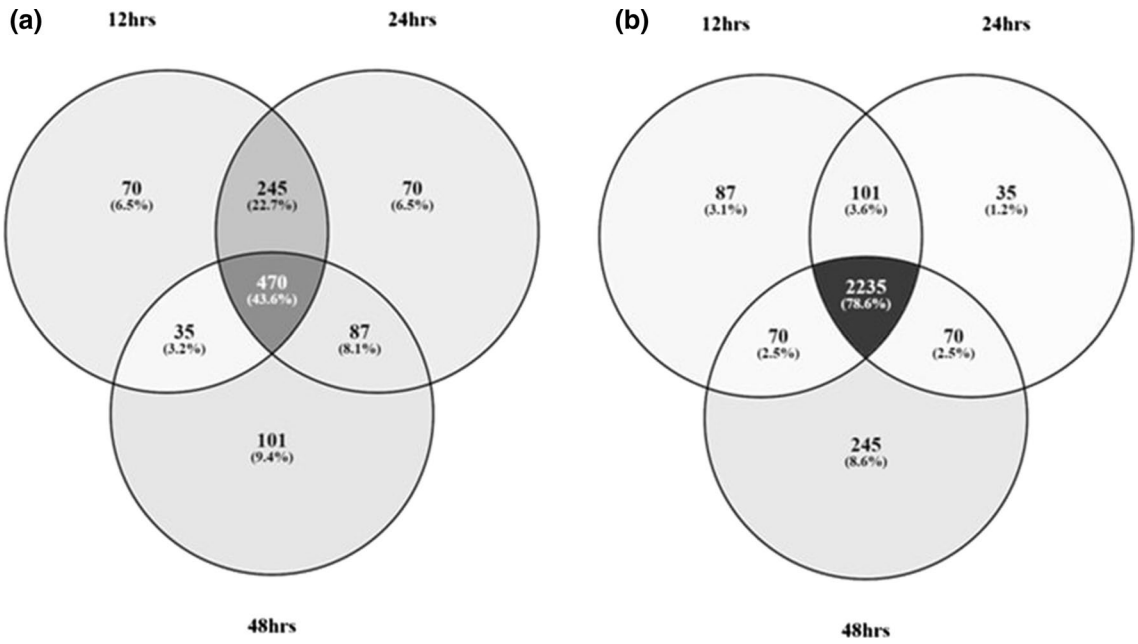

Fig. 1 Comparative analysis of upregulated (a) and downregulated (b) proteins at different time points postinfection in chicken lung tissues

tissues. Differential expression of proteins that are associated with the repair of damaged lung tissues, including signal transduction molecules, kinases, and other biochemical-metabolism-related enzymes, have been reported in dogs infected with influenza virus [14]. In addition, some apoptosis- and tumor-associated proteins (BCL6, FAF1, TBX5, AKAP13, TNFSF10, and TGFB1) were also identified in the chicken lung proteome. We also did GO term analysis of proteins that were exclusively expressed after infection (Table 3). This analysis result provides information on how the disease progresses from onset to outcome in chickens. At 12 h postinfection, cellular homeostasis was primarily affected, whereas at 48 h postinfection, critical pathways such as the influenza A pathway, the chemokine signaling pathway, the Jak-STAT signaling pathway, apoptosis, and the MAPK signaling pathway were activated (Table 3). These results indicate that the virus initially disrupts cellular homeostasis and then activates critical pathways at a later stage of infection.

Molecular pathogenesis of H5N1 infection in chickens

A previous transcriptomics study by our group found that highly pathogenic H5N1 virus induced excessive expression of type I IFNs, cytokines, chemokines, and interferon-stimulated genes (ISGs) in the lung tissues. This atypical expression of immune genes (cytokine storm) might be the cause of the high mortality in chickens [28]. However, information on the pathways activated, constituents of the cytokine storm, and therapeutic strategies against the cytokine storm is lacking for avian species. Intensive molecular studies in humans and human-animal model systems have identified (1) activation of TLR3 and 7, as well as endosome (TLR3 and 7) and cytosolic (RIG-I) pathways, (2) activation of the IL1R signaling pathway, and (3) activation of MVAS/MyD88/TRIF signaling as essential pathways involved in the cytokine storm [38].

To determine whether these pathways are activated and to estimate the levels of expression of immune genes in chicken lung tissues, we applied meta-analysis of lung transcriptome datasets. We utilized previously published microarray datasets from chickens infected with HPAIVs because transcriptomic data can capture the complete gene expression dynamics for a particular condition. We mapped the data for differentially expressed genes in chickens obtained from meta-analysis onto the influenza reference pathways in the KEGG database. Activation of the TLR signaling pathway, RIG I signaling pathway, NOD-like receptor signaling pathway, and JAK-STAT signaling pathway were observed in the chicken lung transcriptome (Supplementary Fig. S2). Similarly, activation of these pathways was evident in the chicken proteome datasets (Fig. 3). Further Bayesian networks (BN) were constructed with prior knowledge, using chicken meta-analysis transcriptome datasets (Fig. 4, Supplementary Fig. S3). The combined *in silico* analysis of transcriptome and proteome datasets confirmed the activation of TLRs, RLRs, NLRs, and Jak-STAT signaling pathways in lung tissues infected with HPAIVs in chickens. The fact that influenza pathogenesis is associated with abnormalities in all of these

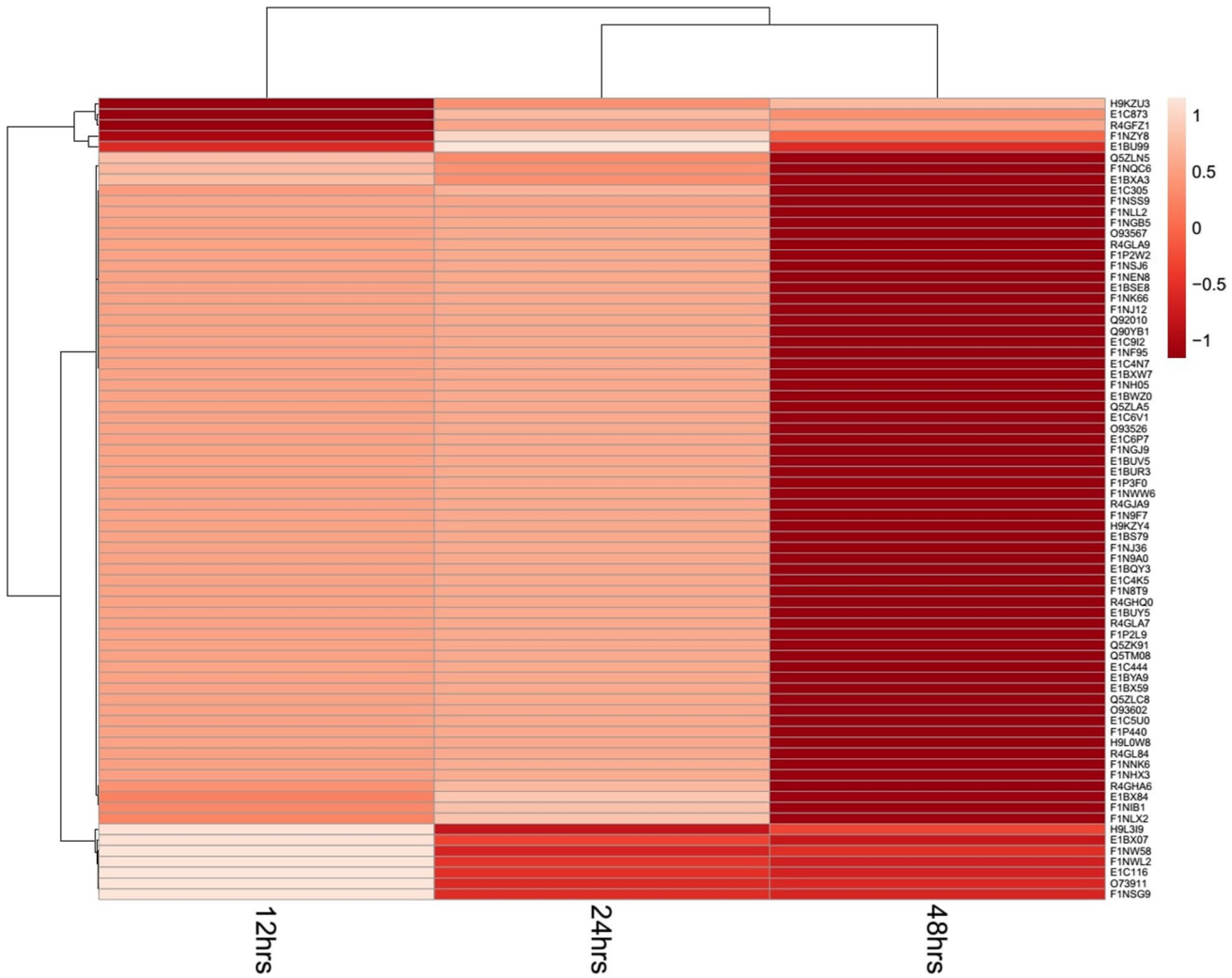

Fig. 2 Heat map of differentially expressed proteins in HPAIV-infected lung tissues. The expression levels are visualized using a gradient colour scheme.

core pathways suggests that they play a central role in the cytokine storm.

Next, we examined the cytokine-storm-responsive genes (i.e., expression level of cytokines, chemokines, and ISGs) as a result of activation of the above-mentioned pathways. A list of cytokine-storm-responsive genes in chickens was compiled based on information in the literature [2]. Data for expression levels (fold change) of these genes were obtained from meta-analysis transcriptome datasets from chickens. Cytokines, chemokines, and ISGs were found to be upregulated in chicken lung tissues, and these may be the basis for the high degree of severity of HPAI H5N1 infection in chickens (Supplementary Fig. S4). In summary, we identified the immune pathways involved in the cytokine storm and identified cytokine-storm-responsive genes in chicken lung tissues infected with HPAIV.

Identification of proteomic determinants of disease pathogenesis in chickens

To identify the main driver or hub proteins responsible for disease pathogenesis, we constructed a protein-protein interaction (PPI) network based on the chicken lung proteome dataset (Fig. 5). Proteins involved in the TLR, RLR, IL1R, and NLR signaling pathways, such as myeloid differentiation primary response protein (MyD88), inhibitor of nuclear factor kappa B kinase subunit beta (IKBKB), interleukin 1 receptor associated kinase 4 (IRAK4), RELA proto-oncogene NF-κB subunit (RELA), and mitochondrial antiviral signaling protein (MAVS), were identified with a high degree of centrality and high betweenness centrality values (Table 4).

The MYD88 gene encodes a cytosolic adapter protein that plays a central role in the innate and adaptive immune

Table 2 Gene Ontology term analysis of commonly upregulated and downregulated lung tissue proteins in chickens infected with HPAI H5N1 virus

GO term	Count	*P*-value
Upregulated proteins		
Golgi apparatus	32	7.70E-04
Cellular homeostasis	21	1.10E-03
Protein heterodimerization activity	12	2.20E-03
Ion homeostasis	18	3.70E-03
Protein domain specific binding	15	4.40E-03
Acid-amino acid ligase activity	11	5.20E-03
Intracellular organelle lumen	50	6.40E-03
Fatty acid transport	4	1.80E-02
Regulation of cell cycle	13	3.50E-02
Vesicle-mediated transport	19	4.20E-02
Positive regulation of protein kinase cascade	8	5.10E-02
Negative regulation of gene expression	16	8.20E-02
Cytoskeleton	35	8.60E-02
Adenyl nucleotide binding	47	1.90E-03
ATP binding	43	4.90E-03
Downregulated proteins		
Adenyl nucleotide binding	236	1.20E-13
ATP binding	222	5.40E-13
GTPase regulator activity	74	4.50E-08
Cytoskeleton	176	1.20E-06
Protein kinase activity	82	9.20E-04
Regulation of small GTPase mediated signal transduction	47	8.10E-06
Regulation of Rho protein signal transduction	25	1.10E-05
Proteinaceous extracellular matrix	51	8.80E-05
Regulation of Ras protein signal transduction	38	1.30E-04
Protein amino acid phosphorylation	89	6.20E-04
Cell death	93	1.30E-03
Enzyme linked receptor protein signalling pathway	49	3.10E-03
Endosome	44	4.10E-03
Intracellular signaling cascade	146	4.30E-03
Positive regulation of gene expression	70	2.50E-02
Calcium ion binding	105	3.20E-02
Negative regulation of translation	8	3.60E-02
Regulation of phosphorylation	56	4.60E-02
JUN kinase binding	3	4.70E-02
Proteolysis	114	7.20E-02
Positive regulation of MAP kinase activity	15	9.60E-02

responses. This protein functions as an essential signal transducer in the IL1R and TLR signaling pathways. These pathways in turn regulate the activation of numerous proinflammatory genes [39]. The IKBKB protein phosphorylates the inhibitor in the inhibitor/NF-κB complex, causing dissociation of the inhibitor and activation of the NF-κB signaling pathway [40]. MAVS acts downstream of the DDX58/RIG-I and IFIH1/MDA5 genes as an essential signal transducer in the beta interferon signaling pathways and contributes to antiviral immunity [41]. RELA/NF-κB is a ubiquitous transcription factor that is involved in several biological processes. This transcription factor is activated through degradation of its specific inhibitor in the cytoplasm; NF-κB moves to the nucleus and activates transcription of specific genes. The NF-κB-p65 complex appears to be involved in invasin-mediated activation of IL-8 expression [42]. Teijaro et al. reported that MyD88 and MAVS are the predominant signaling molecules required for innate immune cell recruitment and the majority of cytokine amplification (i.e., cytokine storm) events in mice infected with influenza virus [38]. Furthermore, they suggested that therapeutic control of the cytokine storm is possible through inhibition of a

Table 3 Functional annotation of proteins expressed at only one time points after infection of chickens with HPAI H5N1 virus

Pathway activation	No. of proteins	*P*-value
12 h postinfection		
Cell cycle	31	2.88E-26
mRNA surveillance pathway	21	4.95E-18
RNA transport	24	2.62E-17
p53 signaling pathway	11	1.22E-07
Pathways in cancer	13	0.0128
24 h postinfection		
Cell cycle	12	1.17E-08
Gap junction	8	7.31E-06
RNA degradation	6	6.13E-05
T cell receptor signaling pathway	7	0.000127
Fc epsilon RI signaling pathway	6	0.000215
ErbB signaling pathway	6	0.000485
Phagosome	4	0.00326
Fc gamma R-mediated phagocytosis	5	0.00529
Chemokine signaling pathway	7	0.00615
B cell receptor signaling pathway	4	0.0112
Neurotrophin signaling pathway	5	0.0141
Natural killer cell mediated cytotoxicity	5	0.0221
Focal adhesion	6	0.0287
48 h postinfection		
RNA transport	47	1.77E-18
T cell receptor signaling pathway	37	6.08E-15
Regulation of actin cytoskeleton	52	1.15E-14
Focal adhesion	54	4.67E-14
Pathways in cancer	70	1.13E-13
ErbB signaling pathway	31	6.41E-12
Adipocytokine signaling pathway	26	6.92E-12
B cell receptor signaling pathway	28	1.91E-11
Neurotrophin signaling pathway	35	3.79E-10
mRNA surveillance pathway	26	6.14E-09
Chemokine signaling pathway	41	7.57E-08
Influenza A	28	1.68E-07
Cell cycle	30	3.95E-07
Jak-STAT signaling pathway	25	1.62E-06
Fc epsilon RI signaling pathway	21	1.86E-06
mTOR signaling pathway	14	2.72E-05
Toll-like receptor signaling pathway	20	0.000377
Apoptosis	18	0.000385
Natural killer cell mediated cytotoxicity	24	0.00139
MAPK signaling pathway	38	0.00297
Leukocyte transendothelial migration	19	0.00374
RIG-I-like receptor signaling pathway	11	0.0039

common pathway downstream of multiple innate pathogen-sensing molecules involved in cytokine amplification. In our study, the identified hub proteins (MyD88, IKBKB, IRAK4, RELA, and MAVS) were all components of the MyD88 and MVAS signaling pathways. Based on the literature, we suggest that successful therapeutic intervention against cytokine storms in chickens should target these proteins to blunt the cytokine amplification. Furthermore, S1P1R agonist therapy may suppress global cytokine amplification in chickens, as it does in mice [38]. However, biological validation of this hypothesis in *in vivo* experiments is needed.

In the Jak-STAT signaling pathway, we found signal transducer and activator of transcription 1 (STAT1), signal transducer and activator of transcription 2 (STAT2), signal transducer and activator of transcription 3 (STAT3), and suppressor of cytokine signaling 3 (SOCS3) proteins to be the main driving proteins in the PPI network (Table 4). However, these proteins had protein probability values ranging from 0.63 to 0.90 and were therefore not evident in our main proteome dataset. The STAT1, STAT2, and STAT-3 proteins are key constituents of the JAK-STAT signaling pathway, play critical roles in the IFN signaling pathway, and are required for a robust IFN-induced antiviral response [43, 44]. SOCS1 and SOCS3 have been reported to be critical regulators of IFN responses through the inhibition of STAT phosphorylation and induction of ISGs through a RIG-I/MAVS/IFNAR1-dependent pathway [45, 46].

We also identified some novel main driver/hub proteins with a very high degree of centrality and high betweenness of centrality values that had not been reported previously to be associated with influenza virus infection in humans, human models, or avian species. These novel hub proteins include hepatocyte nuclear factor 4 alpha (HNF4A), ELAV-like RNA binding protein 1 (ELAVL1), fibronectin 1 (FN1), COP9 signalosome subunit 5 (COPS5), cullin 1 (CUL1), breast cancer type 1 susceptibility protein (BRCA1), catenin beta 1 (CTNNB1), and the FYN proto-oncogene Src family tyrosine kinase (FYN) (Table 4, Fig. 5). The HNF4A protein is a transcriptionally controlled transcription factor that is required for the transcription of alpha 1 antitrypsin, apolipoprotein CIII, transthyretin genes, and HNF1-alpha genes [47]. ELAVL1 is a member of the ELAVL family of RNA-binding proteins and selectively binds AU-rich elements (AREs) found in the 3' untranslated regions of mRNAs. Proteins of the ELAVL family of play a role in stabilizing ARE-containing mRNAs [48]. This gene has been implicated in a variety of biological processes and has been linked to several diseases, including cancer [49].

FN1 binds cell surfaces and various compounds, including collagen, fibrin, heparin, DNA, and actin. FN1 is involved in cell adhesion and migration processes during embryogenesis, wound healing, blood coagulation, host defense, and metastasis [50]. COPS5 is one of the eight subunits of the COP9 signalosome and functions as an important regulator of phosphorylation of p53/TP53, c-jun/JUN, IkappaBalpha/NFKBIA, ITPK1, and IRF8 signaling [51].

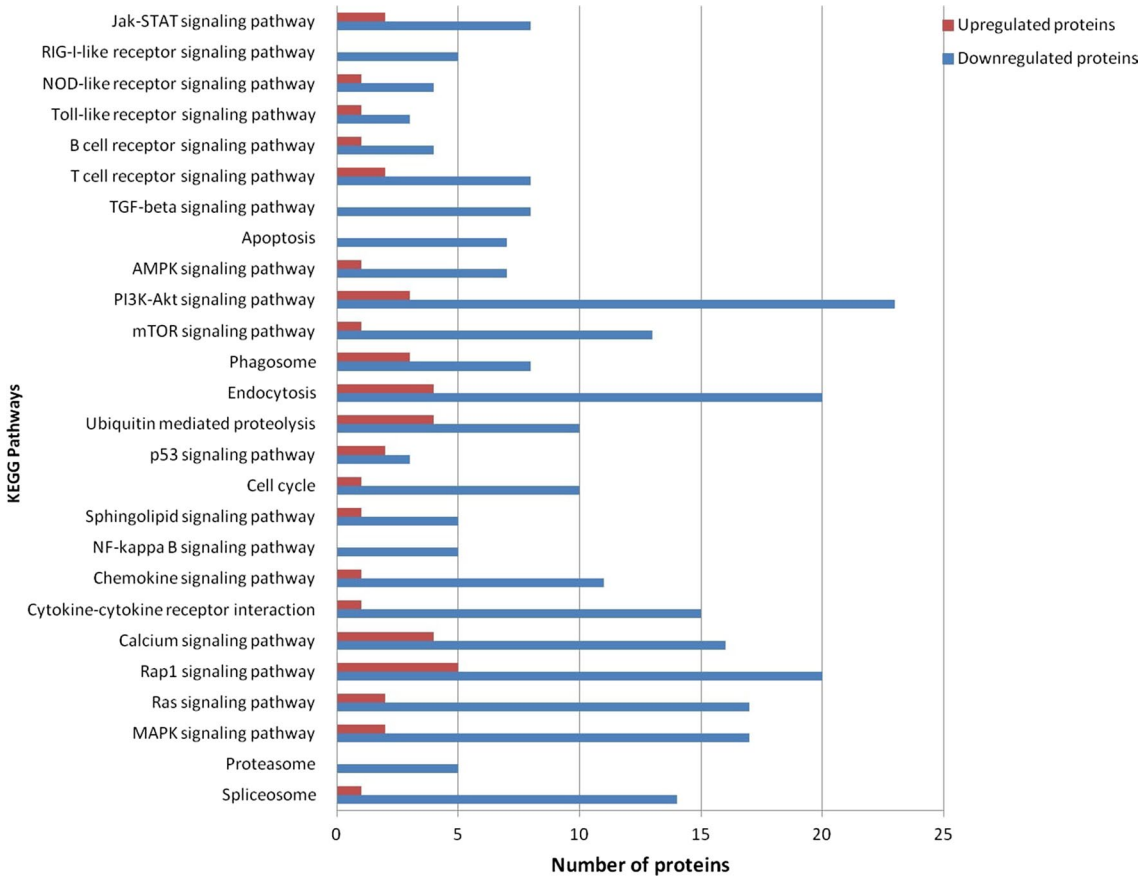

Fig. 3 KEGG pathway analysis of commonly upregulated and downregulated proteins in chicken lung tissues infected with HPAI H5N1 virus

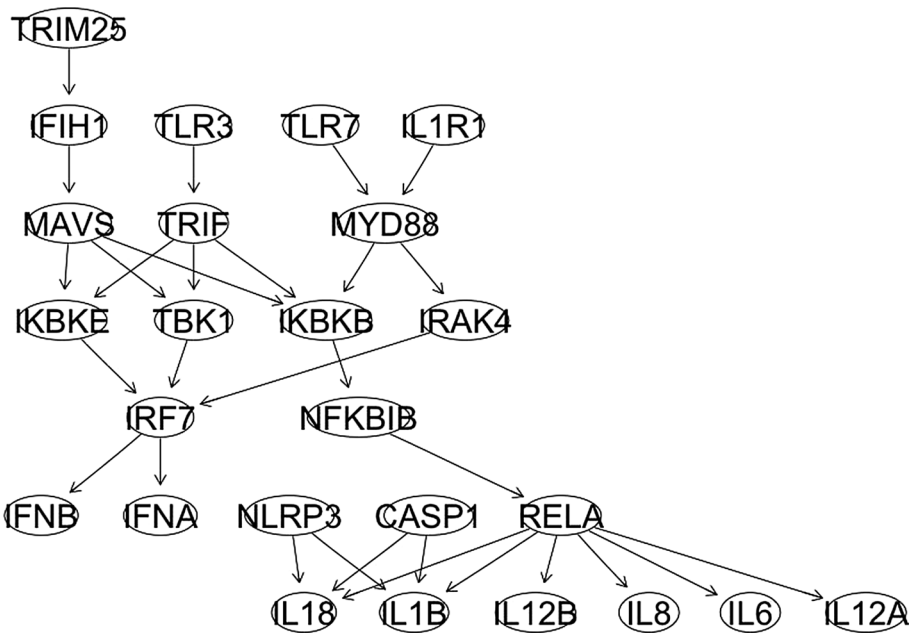

Fig. 4 Bayesian network constructed based on meta-analysis of a transcriptome dataset from chickens, with prior knowledge of the TLR, RLR, IL1R, and NLR signaling pathways in chickens.

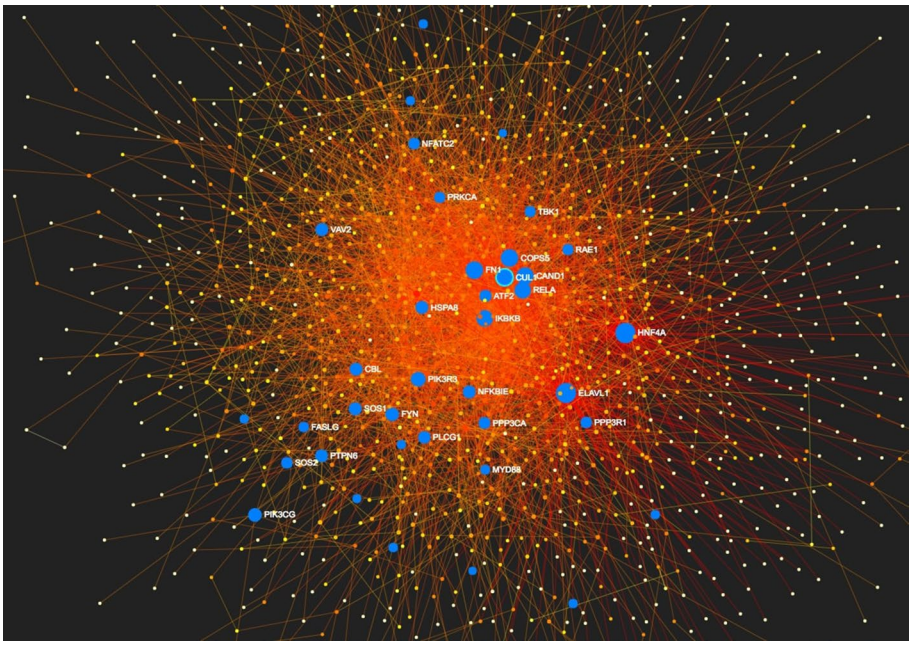

Fig. 5 The protein-protein interaction network of chicken lung tissues infected with HPAI H5N1 virus. The important hub genes involved in the molecular pathogenesis of influenza are highlighted in blue in the PPI network.

CUL1 is a core component of multiple cullin-RING-based SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complexes, which mediate the ubiquitination of proteins involved in cell cycle progression, signal transduction, and transcription [52].

BRCA1 encodes a nuclear phosphoprotein that plays a role in maintaining genomic stability and acts as a tumor suppressor. This protein is involved in transcription, DNA repair of double-stranded breaks, and recombination [53]. The FYN gene encodes a membrane-associated tyrosine kinase that has been implicated in the control of cell growth [54]. In summary, many proteins involved in the TLR, RLR, NLR, and Jak-STAT signaling pathways and other novel proteins were identified as major protein determinants, and these proteins might be linked to disease pathogenesis in H5N1 infections in chickens. However, the critical functional role of these proteins in avian influenza pathogenesis in chickens requires further biological confirmation by *in vivo* and *in vitro* experiments.

Table 4 Hub proteins identified in chicken PPI networks based on degree of centrality and betweenness centrality values

Protein	Degree of centrality	Betweenness centrality
HNF4A	254	254342.21
ELAVL1	254	253172.78
FN1	117	79642.94
COPS5	117	73208.97
CUL1	105	55049.71
CAND1	105	45936.7
CTNNB1	80	75126.54
BRCA1	72	41422.24
FYN	65	35789.72
MYD88	10	2614.04
IKBKB	42	16555.52
RELA	48	23497.55
MAVS	6	1721.53
STAT1	81	46280.33
STAT2	14	1579.16
STAT3	90	66853.77
SOCS3	23	11524.2
IRAK4	8	311.94

Conclusion

We have determined the comprehensive proteome profile of chicken lung tissues infected with HPAI H5N1 virus at different time points postinfection. There are considerable differences in the protein profile at different time points postinfection, as indicated by variations in the levels of expression of certain proteins. Combined analysis of transcriptome and proteome datasets revealed activation of the TLR, RLR, NLR, and JAK-STAT signaling pathways, which are associated with the cytokine storm effect observed in chickens infected with HPAI H5N1 virus. Furthermore, we identified many of the important hub proteins linked to influenza virus pathogenesis in chickens.

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Author contributions Conceived and designed the experiments: A.M., A.A.R. and P.V. Performed the experiments: A.M., S.C., A.A.R, and P.V. Analyzed the data: P.V. and A.M. Contributed reagents/materials/analysis tools: H.V.M., D.D.K., and V.P.S. Wrote the paper: P.V. and A.M. All authors have read and approved the manuscript.

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Availability of data and materials The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium database via the PRIDE partner repository with the dataset identifier PXD010358.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics statement The experiments were approved by the Institutional Animal Ethics Committee of ICAR-NIHSAD (approval no. 68/IAEC/HSADL/12, dated 11.05.2012) and performed under the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India.

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