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DATASET BRIEF

Elucidation of the avian nucleolar proteome by quantitative proteomics using SILAC and changes in cells infected with the coronavirus infectious bronchitis virus

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The nucleolus is a dynamic subnuclear compartment involved in ribosome subunit biogenesis, regulation of cell stress and modulation of cellular growth and the cell cycle, among other functions. The nucleolus is composed of complex protein/protein and protein/RNA interactions. It is a target of virus infection with many viral proteins being shown to localize to the nucleolus during infection. Perturbations to the structure of the nucleolus and its proteome have been predicted to play a role in both cellular and infectious disease. Stable isotope labeling with amino acids in cell culture coupled to LC-MS/MS with bioinformatic analysis using Ingenuity Pathway Analysis was used to investigate whether the nucleolar proteome altered in virus-infected cells. In this study, the avian nucleolar proteome was defined in the absence and presence of virus, in this case the positive strand RNA virus, avian coronavirus infectious bronchitis virus. Data sets, potential protein changes and the functional consequences of virus infection were validated using independent assays. These demonstrated that specific rather than generic changes occurred in the nucleolar proteome in infectious bronchitis virus-infected cells.

Keywords:
Avian coronavirus infectious bronchitis virus / Cell cycle / Microbiology / Protein complexes / Stable isotope labeling with amino acids

The nucleolus is a subnuclear structure formed from complex protein–protein and protein–nucleic acid interactions [1]. The primary function of the nucleolus is in ribosome biogenesis, regulation of the cell cycle and the response to cell stress [2]. The nucleolus may be formed around hub proteins [1], depletion of which can result in alterations in nucleolar architecture [3] and possibly function. The roles the nucleolus play outside its traditional function in ribosome biogenesis have only recently come to light, due in a large part to MS-based approaches to elucidating the nucleolar proteome. The first studies of the human nucleolar proteome revealed 271 and 213 proteins, respectively [4, 5]. The most recent edition of the nucleolar proteome database (NOPdb 3.0) identified over 4500 proteins as being resident within the nucleolus under certain conditions; it estimates at least 80% coverage [6]. It is noteworthy that the majority of studies of the nucleolar proteome have been carried out on human HeLa cells, whereas the only other species investigated in depth is *Arabidopsis thaliana* where 217 proteins with a large degree

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Abbreviations: IBV, infectious bronchitis virus; **N,** nucleocapsid; **SILAC,** stable isotope labeling with amino acids in cell culture

of conservation between the plant and the human proteomes were found [7].

The nucleolus is also a target for cellular and infectious diseases [8]. Many viral proteins localize to this structure. These range from proteins encoded by RNA viruses with purely cytoplasmic replication strategies [9, 10] to DNA viruses with nuclear replication strategies [11]. The nucleolus can change structure in virus-infected cells [12] and this may correspond with a change in proteome [13]. A recent quantitative analysis of the nucleolar proteome isolated from HeLa cells infected with the nuclear replicating DNA virus adenovirus identified 351 proteins, of which 24 proteins showed at least a twofold change in abundance, compared with nucleoli from mock-infected cells [14]. The precise interactions of viruses with the nucleolus are largely unknown [13], particularly puzzling is the case of the RNA viruses that have cytoplasmic replication strategies [9]. For example, in one such virus, avian infectious bronchitis virus (IBV), the virally encoded nucleocapsid (N) protein localized to the nucleolus in a cell cycle-dependent manner [15] and contained appropriate targeting motifs [16–18].

Elucidation of the avian nucleolar proteome would provide a useful data set. Similarly, how the nucleolus responds to RNA virus infection would provide a useful comparator with the recent analysis of the nucleolar proteome in cells infected with a DNA virus [14]. This would help determine whether any general principles can be established, despite different replication strategies. One of the difficulties in studying the avian nucleolus is the lack of appropriate and extensive immunological reagents.

In this study, stable isotope labeling with amino acids in cell culture (SILAC) coupled to LC-MS/MS was used to generate a preliminary map of the avian nucleolar proteome in the presence and absence of IBV infection and was conducted by Dundee Cell Products. The cell type used in this study was DF-1 cells, a chicken fibroblast cell line, which we (and others) have used to study IBV *e.g.* [17]. Cells were infected at a multiplicity of infection of 1 as described previously [19]. Nucleolar fractions were prepared 18 h post-infection as described previously [20] and validated by detecting appropriate marker proteins (data not shown). Quantification was performed with MaxQuant version 1.0.7.4 [21], and was based on 2-D centroid of the isotope clusters within each SILAC pair. The generation of peak list, SILAC- and extracted ion current-based quantitation, calculated posterior error probability and false discovery rate based on the search engine results, peptide to protein group assembly, and data filtration and presentation was carried out using MaxQuant. The derived peak list was searched with the MASCOT search engine (version 2.1.04; Matrix Science, London, UK) against a concatenated database combining 80 412 proteins from International Protein Index human protein database version 3.6 (forward database), and the reversed sequences of all proteins (reverse database). The complete methodology has been described previously [20, 22]. Raw data sets used to generate Supporting Information Tables 1 and 2 (described below) can be found on the PRoteomics IDEntifications (PRIDE) database [23] and were uploaded using the PRIDE database convertor tool [24].

Cellular proteins were initially assigned by comparing with the avian genome database, and identified 835 proteins (Supporting Information Table 1). However, this database had poor annotation compared with the human genome, and many of the proteins were unassigned or uncharacterized. Therefore, the human database was used for protein assignment from peptide identification. In addition, candidate proteins were then examined to determine whether they were previously identified in the human nucleolar proteome database, which contains some 4500 proteins with an estimated 80% coverage. Only if both of these criteria were met was the protein then assigned as being present in the avian nucleolar proteome (Supporting Information Table 2). These resulted in a final list of 378 cellular proteins being identified and of these 260 proteins being identified and quantified. One viral protein, the N protein, was identified in the nucleolus with 59.4% coverage (Fig. 1). This had not been previously observed in the nucleolus of IBV-infected DF1 cells, possibly because of the difficulties associated with the indirect immunofluorescence detection of nucleolar proteins [25]. Previous overexpression of an EGFP-tagged N protein (in the absence of IBV infection) in DF1 cells demonstrated the potential cytoplasmic and nucleolar localization of this protein [17]. No other IBV proteins were detected in the nucleolar fraction, and this analysis demonstrated the nucleolar localization of N protein in the context of IBV-infected cells.

Several proteins were selected from the SILAC LC-MS/MS analysis and their abundance and localization investigated in mock and IBV-infected cells using indirect immunofluorescence confocal microscopy (as described in [18, 19]) in order to validate the quantitative proteomic approach (Supporting Information Fig. 1). Selection of these proteins depended primarily on available antibody but also to demonstrate the validity of the SILAC LC-MS/MS analysis for several different cases: Proteins identified by multiple peptides showing either no significant change (HSP70, 1.38-fold increase, identified by 14 peptides) or a significant

MASGKAAGK***TDAPAPVIKLGGPKPPKVGSSGNASWFQAIK***
AK***KLNTPPPKFEGSGVPDNENIKPSQQHGYWR***RQARFKPG
KGGRKPVPDAWYFYYTGTGPAADLNWGDTQDGIVWVAAKG
ADTKSRSNQGTR***DPDKFDQYPLRFSDGGPDGNFRWDFIPL***
NRGRSGR***STAASSAAASR***APSREGSRGR***RSDSGDDLIAR***A
AK***IIQDQQK***KGSRITK***AKADEMAHR***RYCKRTIPPNYR***VDQ***
VFGPRTKGKEGNFGDDK***MNEEGIKDGRVTAMLNLVPSSHA***
CLFGSRVTPK***LQLDGLHLRFEFTTVVPCDDPQFDNYVKIC***
DQCVDGVGTRPKDDEPKPKSRSSSRPATRGNSPAPRQQRP
KKEKKLKKQDDEADK***ALTSDEERNNAQLEFYDEPKVINWG***
DAALGENEL

Figure 1. Amino acid sequence of the IBV N protein showing peptides (bold and italic face) that were identified in the nucleolar fraction prepared from DF1 cells infected with IBV.

change (HSP90, 4.30-fold increase, five peptides), to a protein identified by a single peptide and large increase (Shroom3 ~50-fold increase). In IBV-infected cells, there was no apparent change in HSP70, whereas HSP90 appeared to have increased punctate localization in the nucleolus, nucleus and cytoplasm. In IBV-infected cells, the nucleolar localization of Shroom3 appeared to increase. Therefore, in general, the indirect immunofluorescence confocal microscopy analysis reflected the observations of the SILAC LC-MS/MS analysis.

Ingenuity Pathway Analysis was used to investigate the data sets to group together any proteins that shared similar functions in order to build an overview of the avian nucleolar proteome (Fig. 2) and potential roles of proteins in infectious and respiratory disease. Predominant functions in the nucleolus included 54 proteins identified as being involved in protein synthesis (p-value, $1.55 \times 10^{-11} - 1.54 \times 10^{-2}$), 37 proteins involved in RNA post-transcriptional modification (p-value, $4.63 \times 10^{-13} - 1.94 \times 10^{-2}$), 38 proteins involved in molecular transport (p-value, $5.70 \times 10^{-7} - 1.94 \times 10^{-2}$), 25 proteins involved in nucleic acid metabolism (p-value, $1.05 \times 10^{-6} - 2.75 \times 10^{-2}$) and 20 proteins involved in DNA replication, recombination and repair (p-value, $1.11 \times 10^{-6} - 2.75 \times 10^{-2}$). Major linked networked functions include those in protein synthesis, gene expression, RNA post-transcriptional modification, DNA replication, recombination and repair (Supporting Information Fig. 2), cellular signaling (Supporting Information Fig. 3), where induction of interferon has been established experimentally in IBV infection [26] and also the network of cell growth and proliferation linked to protein synthesis (Fig. 3A).

For quantitative analysis, the previous investigations using SILAC and LC-MS/MS have applied ratio cutoffs ranging from nearly 1.3- to 2.0-fold. A previous study that investigated changes on nuclear proteins in RSV-infected cells using 2-D gel electrophoresis coupled to MALDI-TOF used a ratio cut off of 2.0-fold for comparison. The more recent investigation of the nucleolar proteome in adenovirus-infected cells compared with mock-infected cells using SILAC coupled to LC-MS/MS as well 2-D gel electrophoresis used a cutoff of twofold to access significant differences. In our study, a 2.0-fold cutoff was also used as a basis for investigating potential proteome changes using Ingenuity Pathway Analysis in order to provide comparison with the previous studies. Selection of a twofold difference gave 179 proteins that showed differences in abundance between nucleoli isolated from IBV-infected cells from nucleoli isolated from mock-infected cells. Despite this, not all nucleolar proteins changed in IBV-infected DF-1 cells. For example, the nucleolar proteins nucleolin and NOP56 and NOP58 were not significantly increased in IBV-infected cells with 1.8-fold, 1.2- and 1.5-fold differences, respectively.

Analysis of the relationship of identified nucleolar proteins by Ingenuity Pathway Analysis suggested perturbations in the nucleolar proteome could have potential effects on cell metabolism. For example, several proteins were directly linked to regulation and/or binding by cyclin A and retinoblastoma protein (Fig. 3A). Also several molecules which are involved in G2/M-phase transition were increased approximately four to fivefold in abundance in the nucleolar proteome in IBV-infected cells including three components of the 14-3-3 complex. Although the interaction between the IBV and the cell cycle has been characterized in several different nonavian cell lines [19, 27, 28], the potential interaction between the IBV and the host cell in nonavian cell lines has been reported to be different to what occurs in avian

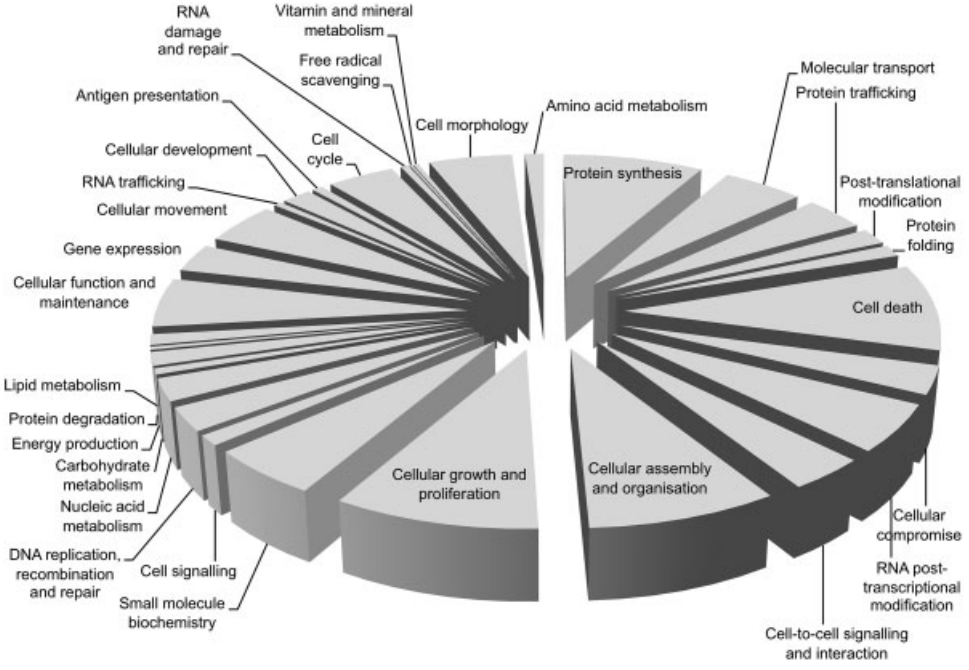

Figure 2. Classification of cellular proteins in nucleolar fractions prepared from DF1 cells according to their assigned fraction and biological function. Formal descriptions of the different assigned functions are summarized in Supporting Information Table 3.

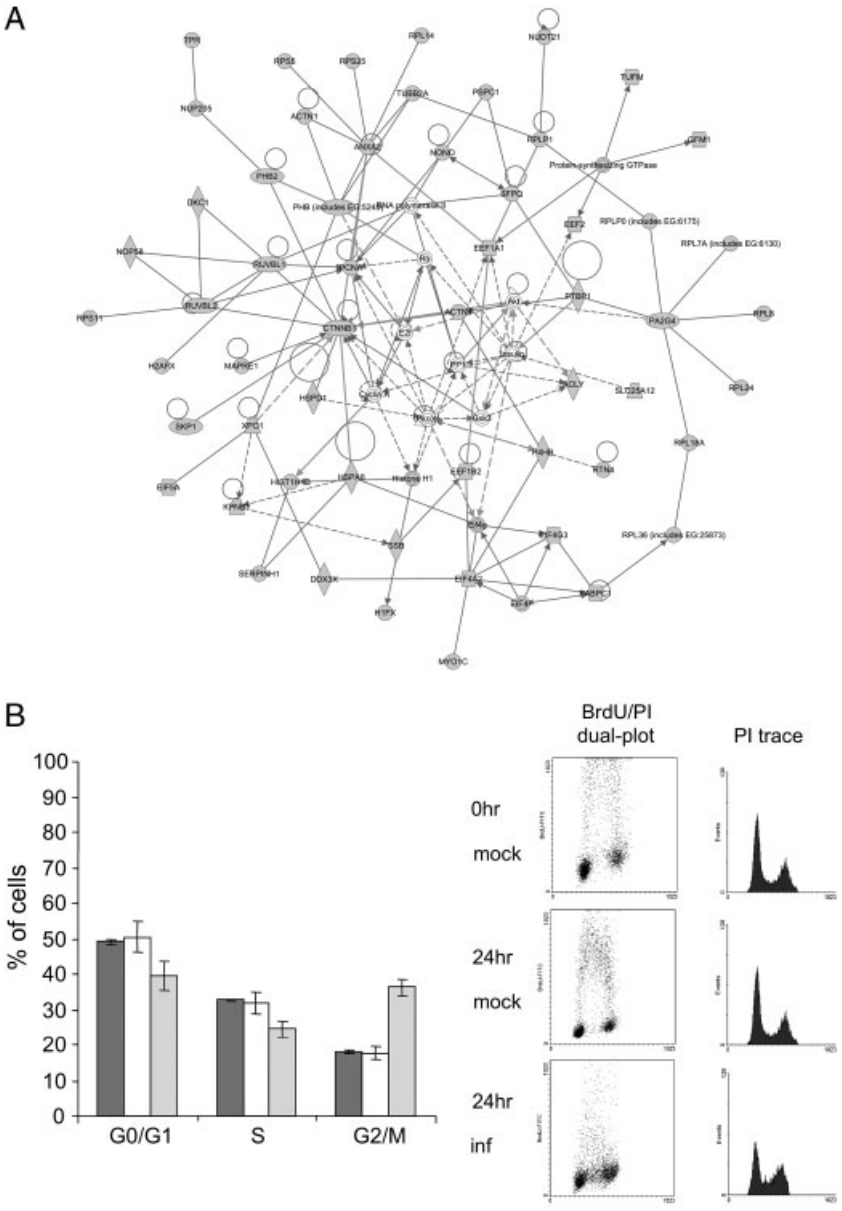

Figure 3. (A) Ingenuity Pathway Analysis of proteins predominately associated with protein synthesis, gene expression, DNA replication, recombination and repair, cellular assembly and organization and RNA post-transcriptional modification in the nucleolar proteome. The shapes are indicative or the molecular class (*i.e.* protein family). Proteins highlighted in grey shading were identified in the LC-MS/MS analysis. Proteins highlighted in clear were not identified in the LC-MS/MS analysis but were linked to the identified protein by examination of the Ingenuity Pathway Analysis curated database describing protein:protein interactions. Examples such as cyclin A are present in the human nucleolar proteome [6], whereas retinoblastoma (Rb) is not, but may never the less be linked to nucleolar pathways as suggested. Lines connecting the molecules indicate molecular relationships. There are two line styles: dashed lines indicate indirect interactions and solid lines indicate direct interactions. The style of the arrows indicates specific molecular relationships and the directionality of the interaction (X acts on Y). (B) Analysis of the proportion of cells in different stages of the cell cycle in mock at 0 h (dark grey shade) and 24 h (clear) and IBV-infected cells at 24 h (light grey). Also shown are examples of raw data output for the cell cycle analysis based on staining with BrdU/PI or PI only.

cells [29]. Therefore, to investigate whether the cell cycle was altered in IBV-infected avian cells and to validate network pathway analysis, dual label flow cytometry was used to accurately compare the proportion of cells in different stages of the cell cycle between mock and infected cells [19, 30]. The experiment was conducted in triplicate, on three occasions and the data indicated that in IBV-infected cells there was a significantly greater proportion of cells in the G2/M stage compared with mock-infected cells (Fig. 3B).

These data sets (both raw *e.g.* Supporting Information Table 1) and processed (Supporting Information Table 2) provide the first solution of the avian nucleolar proteome. The use of SILAC allowed the perturbation of the nucleolar proteome in IBV-infected cells to be examined and compared with mock-infected cells. The data indicated that, in general, there was an increase in nucleolar proteins in IBV-infected cells that was validated by indirect immunofluorescence confocal microscopy. This technique was independent of purification of proteins from the nucleolus and thus provided distinct validation of LC-MS/MS data. Functional predictions such as cell cycle aberrations could be made in terms of bioinformatic analysis of the nucleolar proteome and this was validated using an appropriate assay. Overall, the study demonstrates how SILAC coupled to LC-MS/MS can be used to study changes in the proteome of a cellular compartment (in this case, the nucleolus) in virus-infected cells.

Raw data sets used to generate Supporting Information Tables 1 and 2 have been uploaded to the PRIDE database.

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