REVIEW

Nucleolar proteomics and viral infection

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Recent advances in proteomics have been combined with traditional methods for isolation of nucleoli from mammalian and plant cells. This approach has confirmed the growing body of data showing a wide role for the nucleolus in eukaryotic cell biology beyond ribosome generation into many areas of cell function from regulation of the cell cycle, modulation of the cell stress response to innate immune responses. This has been reflected in the growing body of evidence that viruses specifically target the nucleolus by sequestering cellular nucleolar proteins or by targeting viral proteins to the nucleolus in order to maximise viral replication. This review covers those key areas and looks at the latest approaches using high-throughput quantitative proteomics of the nucleolus in virus infected cells to gain an insight into the role of this fascinating compartment in viral infection.

Received: April 16, 2010
Revised: May 24, 2010
Accepted: May 27, 2010

Keywords:
Microbiology / Nucleolus / Virology

1 Introduction

Although the nucleolus is traditionally the site of rRNA synthesis and ribosome assembly, over the last 20 years or so it has become clear that the nucleolus plays a role in a wide range of important cellular processes [1–3]. For example, the nucleolus functions as a stress sensor; in UV irradiated cells only nucleolar damage induced p53 stabilisation and not DNA damage itself [4]. The tumour suppressor protein, ARF, is stored in the nucleolus and is part of a complex pathway involving nucleolar antigen B23.1 and HDM2 to control p53 levels [5–9]. In cell cycle control, the RENT complex helps govern cell cycle progression [10–13]. On a wider level of global control, SUMO specific proteases SENP3 and SENP5 are primarily nucleolar localised [14, 15]. Even classical nucleolar antigens like nucleophosmin (B23.1), which have been shown to play a role in rRNA processing, have wider roles. For example, B23.1 helps to control centrosome duplication during mitosis [16–20]. Thus, the nucleolus plays a more extensive role than that of ribosome biogenesis.

Given this information, it seems natural to attempt to understand the functions of the nucleolus in terms of resident proteins. This proteomic approach to understanding the role of the nucleolus in cell function has relied on efficient, robust purification of nucleoli from standard cell lines and the advent of high-throughput quantitative proteomics.

2 Isolating nucleoli

Nucleoli can be isolated from cells in a relatively straightforward fractionation experiment developed some time ago [21–23]. Cells are hypotonically swollen and the cytoplasm is sheared off using a dounce homogeniser. Next, the nuclei are enriched by centrifugation over a sucrose cushion. Purified nuclei are then gently sonicated to release the nucleoli, which are further separated from the nucleoplasm by centrifugation through another sucrose cushion. With care and practice, highly enriched nucleoli can be isolated in this manner. A key aspect of this protocol is the testing of the various fractions to ensure the high-quality enrichment

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Abbreviations: ActD, actinomycin D; **HVS,** Herpesvirus Saimiri; **HSV-1,** herpes simplex virus 1; **KSHV,** Kaposi's sarcoma associated herpesvirus; **PRRSV,** porcine reproductive and respiratory syndrome virus; **SILAC,** stable isotope labelling with amino acids in cell culture; **UBF,** upstream binding factor

of nucleoli from the cytoplasm and nucleoplasm. This is normally monitored by Western blotting of the fractions with antiserum to classical components of each fraction, *e.g.* nuclear lamin should only be present in the nucleoplasmic fraction, nucleolin enriched in the nucleolar fraction.

Coupling this fractionation with the latest high-throughput techniques has been pioneered by groups lead by A. Lamond, J. Diaz and M. Mann [24–27]. This has culminated in a comprehensive data set covering some 4000 members of the mammalian nucleolar proteome (http://www.lamondlab.com/NOPdb3.0). In parallel, the nucleolar proteome of the model plant system Arabidopsis has been analysed [28] in similar detail using isolation techniques specific for plant cells covering just over 200 proteins (http://bioinf.scri.sari.ac.uk/cgi-bin/atnopdb/home).

What these studies have revealed is that nucleoli contain many proteins whose primary functions are not related to ribosome synthesis or biogenesis. As stated earlier, there are a range of proteins present whose primary functions range from cell cycle control to p53 regulation [3]. Comparison of the plant and mammalian nucleoli reveals intriguing differences that presumably reflect different evolutionary pathways. For example, very few proteins involved in mRNA transport are found in the mammalian cell nucleolus, whereas a number of mRNA transport factors are found in the plant cell nucleolus [28].

3 Dynamic proteomics of the nucleolus

The dynamic nature of the nucleolar proteome was illustrated by coupling high-throughput proteomic analysis with stable isotope labelling with amino acids in cell culture (SILAC) [26]. This technique compares two populations of cells: one labelled with normal ^{12}C and ^{14}N arginine and lysine; the other labelled with ^{13}C and ^{15}N labelled arginine and lysine for example. The cells grown in heavy labelled media are treated in this case with actinomycin D (ActD) which inhibits rRNA synthesis. The nucleoli are isolated from normal cells with normal amino acids and combined with nucleoli isolated from heavy labelled cells that have been exposed to ActD. These combined samples are separated by 1-D electrophoresis and the gel divided into a number of slices. Normally, the more slices the lane is divided into the more sensitive and accurate the process of detection, identification and quantitation becomes because of reduction in sample complexity. Each slice is in-gel digested with trypsin and the peptides are analysed by LC-MS/MS. Since each peptide is chemically identical, both the light and heavy peptides are identified, detected and quantified together by MS/MS allowing changes in abundance to be inferred. After suitable analysis, the data returned represents a ratio or fold change in the quantity of each identified protein. That is to say this approach determines the relative enrichment or depletion of proteins in the nucleoli (or indeed any appropriately paired sample).

Using this technique has enabled a proteomic analysis of the changes in the nucleolar proteome over time in response to different treatments. For example, after ActD treatment, nearly a third of just over 500 nucleolar proteins analysed experienced at least a twofold change in their abundance. The types of proteins affected were as widespread as the nucleolar proteome itself, covering DEAD box helicases to snRNP proteins [26]. Indeed, the authors conclude that the nucleolar proteome is not a fixed object but one that comprises overlapping sets of proteins that are present depending on the conditions of the cell at any one time.

A larger scale development of this approach uses triple labelling of cells to examine the content of the nucleolus, nucleoplasm and cytoplasm of cells in response to DNA damage. In this experiment, cells were grown in either light ($^{12}C^{14}N$ L-arginine and L-lysine), or medium (L-arginine-$^{13}C_6$ $^{14}N_4$ and L-lysine-$^{2}H_4$) or heavy (L-arginine-$^{13}C_6$-$^{15}N_4$ and L-lysine-$^{13}C_6$-$^{15}N_2$) growth media. The cells were fractionated into nucleolus, nucleoplasm and cytoplasm and the fractions were recombined such that each subcellular component had a different isotopic label. In this way, proteins could be identified that were enriched in one compartment or present in two compartments or even distributed between all three. In the next step, cells were exposed to etoposide and the process repeated. In this manner, proteins that changed subcellular compartments in response to DNA damage could be tracked. In the case of nucleolar antigens, it seems that DNA damage increases the concentration of nucleolar antigens in the nucleolus relative to other compartments.

Proteomic analysis of the nucleolus confirms independent evidence that a wide range of proteins are present in this structure. In many cases, the nucleolus seems to act as a temporary storage or sequestration site that presumably facilitates a rapid response to stimuli.

4 Viruses and the nucleolus

Recently, we have become interested in the role of the nucleolus in viral infections. Over the last 10 years or so, it has become clear that the majority of viruses disrupt nucleolar function and/or they make proteins that are directed to the nucleolus. Perhaps, the defining viral nucleolar interaction is the observation that HIV uses the nucleolus to traffic its mRNA from the nucleus to the cytoplasm [29]. Indeed, the interaction between HIV and the nucleolus has been shown to be critical since inhibiting the trafficking of HIV mRNA through the nucleolus effectively ablates viral replication [30–33]. From this body of work, it has emerged that modified T cell lines that inhibit the virus's ability to effectively use the nucleolus as a trafficking pathway are resistant to HIV infection.

This mRNA export strategy has recently been shown to be the case for a very distinct virus, Herpesvirus Saimiri (HVS) [34]. In both cases, the reasons for using the nucleolus are clear – both viruses rely on mRNA that is not

suited for efficient transport *via* normal mRNA export systems. In the case of HIV, incomplete splicing of viral transcripts make them a target for nonsense-mediated decay and in the case of HVS most of the transcripts are intronless and would not therefore enter the normal mRNA export pathways, which are closely linked to splicing. What is intriguing is that both viruses have hijacked the nucleolus as a suitable (or even necessary) staging post for the export of their ''aberrant'' mRNA.

A number of reviews have highlighted that nucleolar interactions have important implications in the many distinct virus life cycles [35–38]. For example, a large number of virus-encoded proteins traffic to and from the nucleolus. In addition, numerous host cell nucleolar proteins are redistributed to other cellular localisations during the virus replication cycle. However, the implications of these nucleolar modifications on the virus replication and host cell function have yet to be fully elucidated. Here, we highlight several examples that nucleolar proteins play essential roles in multiple steps of the virus replication cycle, from transcriptional regulation and RNA processing to virus entry and egress (summarised in Fig. 1).

5 DNA viruses

A large number of viruses with DNA genomes have been shown to interact with nucleolus, and this perhaps is not surprising as most DNA viruses replicate in the nucleus. For example, a genome-wide screen of three distinct herpesviruses, herpes simplex virus 1 (HSV-1), cytomegalovirus (CMV) and Epstein–Barr virus (EBV), has shown that at least 12 herpesvirus-encoded proteins specifically localise to the nucleolus [39], which are implicated in many aspects of the herpesvirus life cycle. Therefore a number of proteomic studies are currently being undertaken to study changes, in a global context, within the nucleolar proteome during virus infections.

A significant area of virus biology currently being investigated is the role of viral proteins that traffic through the nucleolus. For example, a number of HIV proteins that traffic through the nucleolus have been implicated in virus mRNA processing [40–43]. Similar observations have also been made in herpesviruses. Initial studies utilising the prototype γ-2 herpesvirus, HVS, demonstrated that the HVS nucleolar trafficking ORF57 protein induces nucleolar redistribution of the host cell human TREX proteins, which are involved in mRNA nuclear export [34]. Intriguingly, ablating ORF57 nucleolar trafficking led to a failure of ORF57-mediated viral mRNA nuclear export [34]. The precise role of this nucleolar sequestration is yet to be determined, but possible effects on viral mRNA/protein processing and viral ribonucleoprotein particle assembly are currently being investigated. This property may also be conserved in other ORF57 homologues as recent analysis has shown that the ORF57 protein from Kaposi's sarcoma associated herpesvirus (KSHV) also dynamically traffics through the nucleolus [44]. Moreover, upon the rapid disorganisation of the nucleolus a reduction is observed in virus mRNA nuclear export [45]. The formation of an ORF57-mediated export competent ribonucleoprotein particle within the nucleolus may also have implications for the translation of viral mRNAs. For example, it has recently

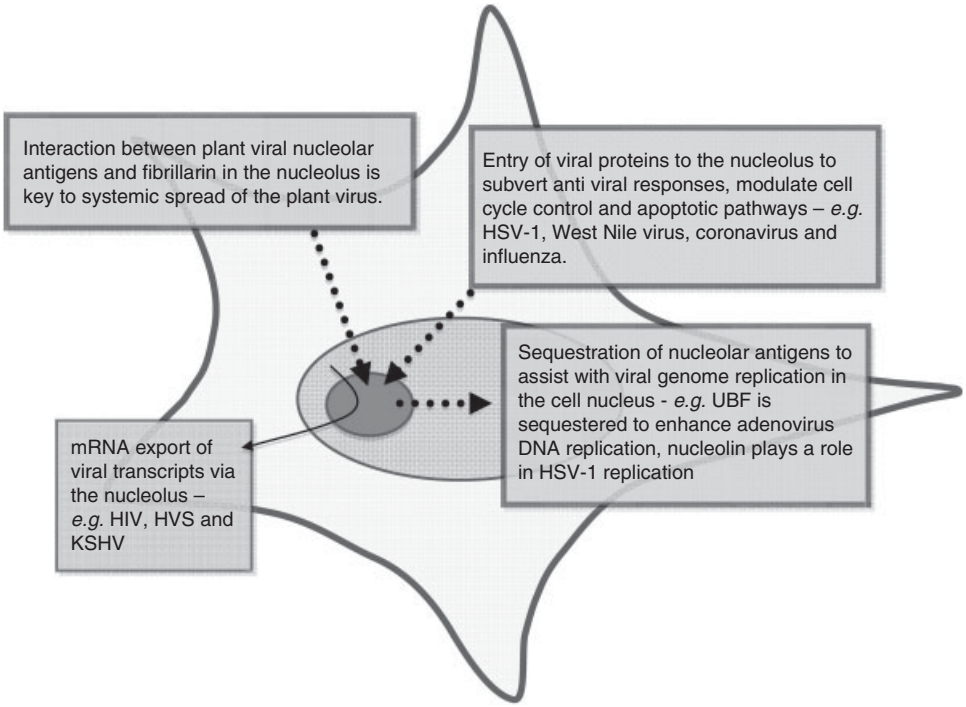

Figure 1. A schematic diagram of the cell with the nucleolus in dark green at the centre. This presents an overview of some of the role of the nucleolus in the life cycle of different viruses. Some viral proteins are directed to the nucleolus to aid viral spread either directly or by affecting apoptotic pathways for example. Some cellular nucleolar antigens are sequestered to aid viral replication and some viruses create a viral mRNA export pathway *via* the nucleolus.

been demonstrated that the cellular nucleo-cytoplasmic shuttle protein, PYM, which is involved in translation enhancement, is redistributed to the nucleolus in the presence of the KSHV ORF57 protein [46]. This interaction effectively enhances the translation of the predominantly intronless transcripts made by KSHV.

A second area of virus replication where nucleolar proteins are sequestered involves the replication of the virus DNA genome. For example, we and others have observed that nucleolar antigens upstream binding factor (UBF) and B23.1 are both sequestered into adenovirus DNA replication centres where they promote viral DNA replication [47–50]. Similarly, in HSV-1 infected cells, a number of nucleolar proteins including nucleolin and UBF are recruited into viral DNA replication centres [51, 52]. These are specific sites where replication and encapsidation of the HSV-1 genome occurs. Evidence suggests that sequestration of UBF is essential for viral DNA replication as overexpression of tagged version of UBF act in a dominant-negative fashion inhibiting virus DNA replication [52]. Moreover, depletion of nucleolin results in reduced virus gene expression and infectious virion production [53, 54].

In addition to enhancing virus replication, nucleolar proteins are redistributed to alter cellular pathways during infection. For example, the nucleolar targeted HSV-1 Us11 protein has been shown to interact with HIPK2, which plays a role in p53-mediated cellular apoptosis and also participates in the regulation of the cell cycle. This interaction alters the subcellular localisation of HIPK2 and protects against HIPK2-mediated cell cycle arrest [55]. In contrast, the cellular protein, PICT-1, can sequester the virally encoded apoptosis suppressor protein, KS-Bcl-2 protein, from the mitochondria into the nucleolus to downregulate its anti-apoptotic activity [56]. In animal models, nucleolar protein ARF has been show to play a protective role in innate immune responses to viral infection [57].

6 RNA viruses

The interaction of many of the RNA viruses with nucleolus on first inspection was unusual [58]. In general, with the exception of influenza virus and several others, this group of viruses employs cytoplasmic replication strategies with all of the viral RNA synthesis and assembly occurring on membrane-bound structures in the cytoplasm. Nevertheless, for many of these viruses examples can be found of viral proteins localising to the nucleolus with potential alterations in the nucleolar architecture and proteome.

One of the most well-characterised examples in terms of localisation and functional relevance is with the nidovirus nucleocapsid (N) protein. The *nidovirales* are a group of viruses that incorporates the positive strand RNA viruses coronaviruses and arteriviruses and have similar genomic organisation and replication strategies. The N protein is a phosphoprotein that complexes and encapsidates the viral genomic RNA [59] and is therefore crucial to virus assembly. However, both the coronavirus [60] and arterivirus [61] N proteins localise to the cytoplasm and nucleolus during virus infection, and with the coronavirus N protein this may be cell cycle dependent [62]. For both proteins, nucleolar localisation signals and nuclear export mechanisms have been characterised [63–66].

Functionally, the nucleolar localisation of these proteins may be important for the virus life cycle. Blocking the nuclear export of the equine arteritis virus protein with the CRM1 dependent inhibitor leptomycin B resulted in the immediate retention of N protein in the nucleus and nucleolus, suggesting that the protein shuttles between the nucleus and the cytoplasm prior to its role in virus assembly [65]. Live cell imaging analysis of fluorescently tagged N proteins coupled to FLIP and FRAP photo-bleaching analysis supported these observations [67, 68]. More definitively, in the context of a recombinant infectious virus, mutation of the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) N protein NoLS resulted in the retention of N protein in the cytoplasm, abrogation of nucleolar localisation and attenuation of virus replication [69, 70]. In both infection in cell culture and *in vivo*, the genotypic alterations to the NoLS in the infectious recombinant virus were repaired and selected for through random mutation with the resulting phenotypic restoration of cytoplasmic and nucleolar localisation of the N protein and resulting of wild-type pathogenicity [69]. Another set of examples can be found in the *filoviridae* where there are numerous examples of West Nile virus and dengue virus interacting with the host cell nucleolus [71–73]. Indeed, in the case of West Nile virus, there is evidence that the interaction with the nucleolus affects p53 stability through the ARF/HDM2/p53 pathway [73]. Some RNA virus proteins that localise to the nucleolus have also been shown to interact with nucleolar proteins such as nucleolin [74] and fibrillarin [75]. For the plant RNA umbraviruses, interaction with the nucleolus and nucleolar proteomes was shown to be crucial for systemic infection [76, 77]. Together, these results clearly demonstrate the functional importance of the localisation of RNA virus proteins to the nucleolus during the virus life cycle and that abrogation of this interaction can be used in the generation of live-attenuated recombinant vaccines. This has been most clearly demonstrated with the work on PRRSV by Pei *et al.* [70]. From previous studies, mutant PRRSV that encoded N proteins with defective NLSs illustrated that wild-type phenotypes were rapidly selected for and restored. Pei *et al.* generated NLS reversion resistant mutants. These progeny viruses were genetically stable for at least 20 passages in cell culture. More importantly, work in swine demonstrated that infection with the mutant viruses resulted in less viremia and of lower titre and shorter duration than wild-type virus. More importantly, there was increased production of neutralising antibodies associated with the mutant viruses. This then is an ideal live attenuated vaccine candidate, in that the virus causes less or no disease

but still stimulates the immune system. It would be interesting to determine whether viruses with N protein NoLS mutations also confer protection when animals are challenged with wild-type virus.

The localisation of RNA virus proteins to the nucleolus and their interactions with nucleolar proteins may also have consequences on nucleolar function [78]. Certainly, at a gross level, nucleolar morphology can change during RNA virus infection [79]. As a first stage in investigating this, high-throughput quantitative proteomics using SILAC have been applied by us to examine the nucleolar proteome in RNA virus infected cells focusing on the avian coronavirus [80] and influenza A virus (unpublished data). Strikingly, very similar to the findings recently reported by us for the DNA virus adenovirus [81], global changes to the nucleolar proteome do not occur and are restricted to selected nucleolar proteins. This suggests that the nucleolus may be broadly robust during virus infection despite the localisation of exogenous (viral) proteins and sequestration of cellular nucleolar proteins by viral infection.

7 Nucleolar proteomics and viral infection

Given the many roles of the nucleolus in the cell life cycle, including its role as a stress sensor, it would seem reasonable that comprehensive unbiased analysis of the nucleolar proteome would yield interesting data. We recently showed that SILAC based high-throughput quantitative proteomics could be used to examine the nucleolus of human cells infected with adenovirus. Human adenoviruses are dsDNA viruses with a genome of approximately 360 000 bp condensed within an icosahedral protein capsid. Viral DNA and proteins enter the nucleus of the cell within an hour of attachment; indeed, we have shown that at least one viral capsid protein (protein V) is delivered to the nucleolus at this stage implying that adenovirus interaction with the nucleolus appears to be throughout the life cycle of the virus [82]. Viral replication is primarily within the nucleus of the cell with the cell eventually succumbing 24–36 h later, releasing up to 10 000 infectious new virus particles. It has been known for some time that adenovirus infection has a profound effect on the processing and export of rRNA as well as affecting nucleolar antigens [83]. More recently, we and others have reported on adenovirus proteins that are directed to the nucleolus and on the sequestration of nucleolar antigens B23.1, B23.2, nucleolin and UBF into viral replication centres [47, 48, 84–92]. Most notably, we showed that UBF was functionally recruited into viral DNA replication centres apparently without affecting the function of RNA pol I. This finding was significant in that UBF is known to directly regulate the activity of RNA pol I, ultimately affecting the rate of rRNA synthesis [93–97]. Moreover, the sequestration of UBF away from RNA pol I was unique to adenovirus infection – experiments in uninfected cells showing sequestration of UBF to non-nucleolar locations also recruited RNA pol I [94]. This provided us with a unique control when we examined the nucleolar proteome using high-throughput quantitative MS [98].

For this experiment, approximately 10^8 HeLa cells that were grown in heavy labelled media were infected with adenovirus at a multiplicity of infection of 5. After 18 h, the cells were harvested and nucleoli isolated and combined with an equal amount of nucleoli from unlabelled uninfected cells. Our analysis of these samples using high-throughput quantitative approaches quantified ratios for 351 proteins. Of those, just 24 (7%) showed a twofold or greater change in quantitation between uninfected and infected nucleoli. At the same time, we compared samples of isolated nucleoli from infected and uninfected cells using more traditional 2-DE, which confirmed that in fact the samples are very similar overall.

That just 7% of proteins identified showed a two-fold or greater change indicates that viral infection targets a specific subset of nucleolar antigens. By comparison, almost a third of nucleolar antigens show a greater than twofold change when cells are treated with ActD which inhibits rRNA synthesis (see Fig. 2A). What is notable is that direct comparison between the adenovirus data set and the ActD dataset shows no clear correlation (Fig. 2B) – further supporting the case that adenovirus induces effects on the nucleolus distinct from that of a generalised, non-specific shut down of nucleolar function. This fits well with our previous observation that adenovirus infection does not affect rRNA synthesis even 36 h post infection [48].

One important feature of using the nucleolus as a source of proteomic information is that large numbers of proteins are primarily located elsewhere only having a low level presence in the nucleolus. In practice this means that alterations in the localisation of predominantly non-nucleolar antigens is also highlighted, presumably since changes elsewhere impact on nucleolar levels as well.

Another useful observation is that examination of the nucleolar proteome gives a different, complementary view of the effects of viral infection on the cell compared to gene array studies. A very detailed analysis of the effect of viral infection on mRNA expression over time has been performed in HFFF cells. There are, of course, a number of difficulties in comparing the two sets of data, not least because they are in different cell types and the virus replicates with slightly slower kinetics in HFFF cells. In most cases, however, there is no real correlation between the two. Moreover, many proteins we observed to be depleted from the nucleolus are not being depleted as a result of mRNA levels dropping off (at least according to data generated in HFFF cells). Whilst we would ideally analyse proteomic and expression data side by side this comparison does help to illustrate the point that levels of mRNA and protein location studies can provide very different answers. This is more directly shown by our studies on nucleolar antigen UBF which regulates rRNA synthesis. UBF is depleted from the nucleolus as recorded by proteomic and microscopy approaches but overall levels of the protein in the cell are not affected as shown by Western blots [48, 98].

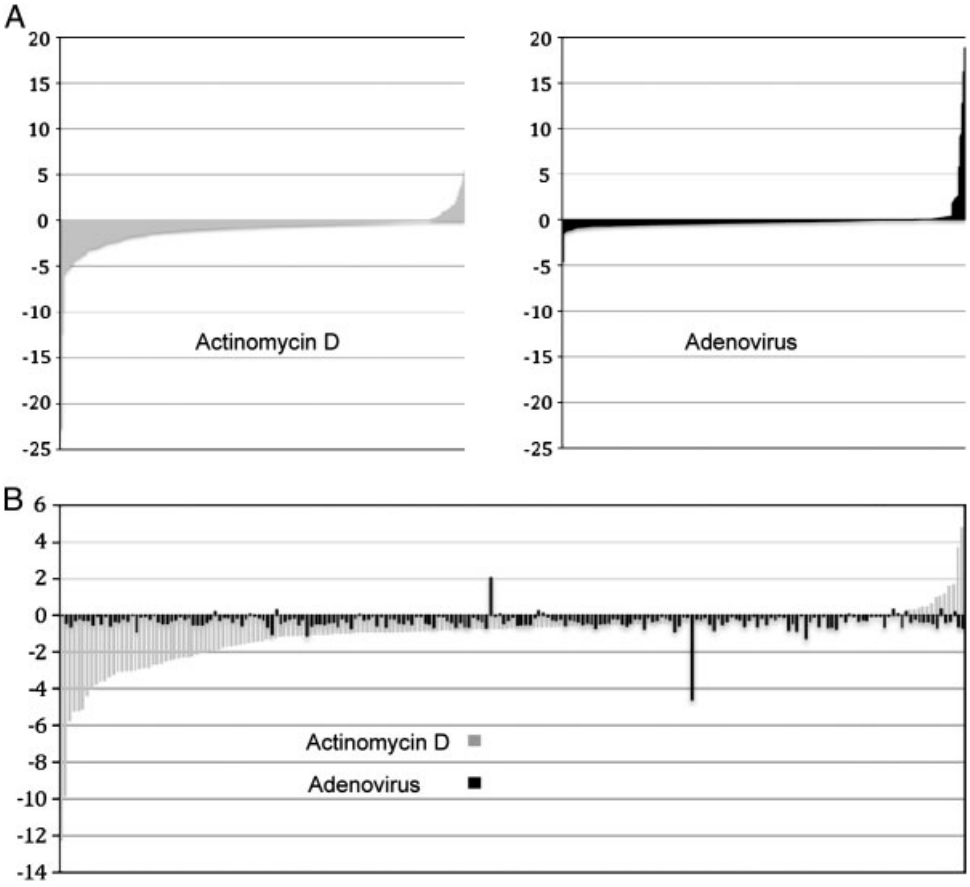

Figure 2. (A) Comparing overall profiles of changes in protein abundance in the nucleolus upon actinomycin D treatment or adenovirus infection. The left hand panel shows the ranges of observed fold changes in the nucleolus of actinomycin D treated cells at 180 minutes post treatment compared to untreated cells. On the right a similar graph shows the ranges of observed fold changes in proteins isolated from uninfected or adenovirus infected cells at 18 hours post infection. Both sets of data were collected in HeLa cells. (B) Comparing the effects of actinomycin D and adenovirus on the nucleolus by aligning proteins. Each pair of bars on the X axis represents an individual protein whose change in abundance in the nucleolus has a calculated ratio in both experiments which were in HeLa cells. In this example, black shows the ratio of change on adenovirus treatment and grey shows how the same protein changes abundance in the nucleolus on actinomycin D treatment.

Building on this, in each of our laboratories, we have begun to examine the nucleolar proteome of a number of other viruses including KSHV, HSV-1, infectious bronchitis virus [80], influenza and respiratory syncytial viruses. Whilst the data is relatively new, there are already encouraging signs that these types of analysis will prove highly informative. For example, there are intriguing similarities in the way the nucleolar proteome is affected by quite distinct viruses. Moreover, validation of findings by immunofluorescence (as was done for adenovirus) has again provided rapid reassurance that the MS/MS data are reflected *in situ*. Indeed, we feel that *in situ* approaches are essential to validate the proteomic data as it provides a rapid and wholly independent means of determining if there are changes in the nucleolar proteome on infection. Most recently, we have begun to examine the changes in the nucleolar proteome over time, which indicates there are distinct changes in the nucleolar proteome at different parts of the virus life cycle.

8 Perspectives

Thus, whilst studies of nucleolar proteome in uninfected cells are relatively well advanced, the application of these approaches to examine the role of the nucleolus in infection is just in its infancy. However, we anticipate that studies currently underway to examine the nucleolus of cells infected with different viruses and in cells expressing individual viral nucleolar antigens in isolation of infection will help us unravel the scope of this structure's role in the replication cycles of important pathogens. In turn, this should also prove highly informative in understanding the scope of influence of the nucleolus on the eukaryotic cell.

The authors thank the Wellcome Trust (DAM Grant No. 083604 and AW Grant No. 086168), Leverhulme Trust (JAH Grant No. RFG/11181) and BBSRC (AW Grant No. BB/G022836/1 and JAH DTG studentship) for funding. The authors also thank Sadie Young for assistance with the data analysis in Fig. 2.

The authors have declared no conflict of interest.

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