Chapter 14
Viruses and the Nucleolus

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14.1 Introduction

The nucleolus is a dynamic sub-nuclear structure with roles in ribosome subunit biogenesis, mediation of cell stress responses and regulation of cell growth (Boulon et al. 2010). The proteome and structure of the nucleolus are constantly changing in response to metabolic conditions, and virus infection represents one of the major challenges to nucleolar function (Greco 2009; Hiscox 2002, 2003, 2007; Hiscox et al. 2010). Viruses are obligate intracellular parasites and rely on the host cell for genome replication, protein expression and assembly of new virus particles. During infection there is a constant war between viruses trying to subvert the host cell and host-mediated anti-viral activity and interaction with the nucleolus is likely to be a key stage in this.

Interaction with the nucleolus is a pan-virus phenomenon and evidence suggests that proteins from many different types of viruses, such as those with DNA, RNA or RNA/DNA (e.g. retroviruses) genomes, encode proteins that can localise to the nucleolus during infection (Table 14.1). These examples include viruses with DNA genomes including the poxviruses, which replicate in the cytoplasm, as well as the herpes and adenoviruses, which replicate in the nucleus. HIV-1, perhaps the classic example of a retrovirus, undergoes an initial replication event in the cytoplasm and then further activity in the nucleus. RNA viruses encompass genomes of single-stranded positive and negative polarity and also double-stranded RNA. Established dogma suggests that positive strand-RNA viral genome synthesis and transcription occur in the cytoplasm. Examples of negative strand RNA viruses can be found, which replicate in the cytoplasm (most of the Mononegavirales) and the nucleus (e.g. influenza viruses).

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Table 14.1 Representative examples of viral proteins from different classes of viruses that localise to the nucleolus

Family	Subfamily	Genus	Virus	Protein/nucleic acid	Reference
Baltimore class I: double-strand DNA					
Adenoviridae	n/a	Mastadenovirus	Human adenovirus type 5 (Ad5)	E4orf4, preMu, pIVa2, preVII, Mu, UXP, protein V	Miron et al. (2009); Lee et al. (2004); Lee et al. (2003); Lutz and Kedinger (1996); Tollefson et al. (2007); Matthews and Russell (1998)
Asfaviridae	n/a	Asfivirus	African swine fever virus (ASFV)	I14L	Goatley et al. (1999)
Herpesviridae	n/a	Mardivirus	Mareks disease virus/ Gallid herpesvirus 2 (MDV)	MEQ	Liu et al. (1997)
Herpesviridae	Alphaherpesvirinae	Simplexvirus	Herpes simplex virus type 1 (HSV-1)	EAP, gamma(1)34.5, ICP0, ICP27, RL1, US11, UL12, UL24, UL27.5, VP22	Leopardi and Roizman (1996); Cheng et al. (2002); Morency et al. (2005); Mears et al. (1995); Salsman et al. (2008); Maclean et al. (1987); Sagou et al. (2010); Lymberopoulos and Pearson (2007); Salsman et al. (2008); Harms et al. (2000)
Herpesviridae	Alphaherpesvirinae	Simplexvirus	Herpes simplex virus type 2 (HSV-2)	UL24, UL3, UL31	Hong-Yan et al. (2001); Yamada et al. (1999); Zhu et al. (1999)
Herpesviridae	Alphaherpesvirinae	Varicellovirus	Bovine herpesvirus 1 (BHV-1)	BICP27	Guo et al. (2009)

Herpesviridae	Betaherpesvirinae	Cytomegalovirus	Human cytomegalovirus (HCMV)	TLR5, TLR7, TLR9, US33, UL29, UL31, UL44, UL76, UL83 (pp65), UL108, UL123 (IEp72)	Salsman et al. (2008); Strang et al. (2010); Arcangeletti et al. (2003)
Herpesviridae	Gammaherpesvirinae	Lymphocryptovirus	Epstein-Barr virus (EBV/HHV-4)	EBNA-5, v-snoRNA1	Szekely et al. (1995); Hutzinger et al. (2009)
Herpesviridae	Gammaherpesvirinae	Rhadinovirus	Kaposi's sarcoma-associated herpesvirus (KSHV)	KS-Bcl02, ORF57	Kalt et al. (2010); Boyne and Whitehouse (2009)
Herpesviridae	Gammaherpesvirinae	Rhadinovirus	Herpesvirus saimiri (HVS)	ORF57	Boyne and Whitehouse (2006)
Poxviridae	Chordopoxvirinae	Leporipoxvirus	Myxoma virus	M184R	Blanie et al. (2009)
Baltimore class II: single-strand DNA					
Circoviridae	n/a	Cirovirus	Porcine circovirus type 1 (PCV1)	Cap	Finsterbusch et al. (2005)
Geminiviridae	n/a	Begomovirus	Tomato leaf curl Java virus (TYLCV)	CP	Sharma and Ikegami (2009)
Geminiviridae	n/a	Begomovirus	Tomato yellow leaf curl virus (TYLCV)	CP	Rojas et al. (2001)
Parvoviridae	Parvovirinae	Dependovirus	Adeno-associated virus	AAP, Rep, Capsid	Sonntag et al. (2010); Wistuba et al. (1997)
Parvoviridae	Parvovirinae	Parvovirus	Minute virus of mice (MVM)	Viral DNA	Walton et al. (1989)
Baltimore class III: double-strand RNA					
Reoviridae	n/a	Orthoreovirus	Avian reovirus	sigmaA	Vazquez-Iglesia et al. (2009)
Baltimore class IV: positive-sense, single-strand RNA					
n/a	n/a	Pomovirus	Potato mop top virus (PMTV)	TGB1	Wright et al. (2010)

(continued)

Table 14.1 (continued)

Family	Subfamily	Genus	Virus	Protein/nucleic acid	Reference
n/a	n/a	Umbravirus	Groundnut rosette virus (GRV)	ORF3	Ryabov et al. (1998)
Arteriviridae	n/a	Arterivirus	Lactate dehydrogenase-elevating virus (LDV)	N	Mohammadi et al. (2009)
Arteriviridae	n/a	Arterivirus	Porcine respiratory and reproductive virus (PRRSV)	N	Rowland et al. (1999)
Bromoviridae	n/a	Cucumovirus	Cucumber mosaic virus (CMV)	2b, Capsid	Gonzalez et al. (2010); Lin et al. (1996)
Coronaviridae	Coronavirinae	Alphacoronavirus	Alphacoronavirus 1/ Transmissible grastroenteritis virus (TGEV)	N	Wurm et al. (2001)
Coronaviridae	Coronavirinae	Betacoronavirus	Murine coronavirus/ Mouse hepatitis virus (MHV)	N	Wurm et al. (2001)
Coronaviridae	Coronavirinae	Betacoronavirus	Severe acute respiratory syndrome coronavirus (SARS-CoV)	3b, N	You et al. (2005); Yuan et al. (2005)
Coronaviridae	Coronavirinae	Gammacoronavirus	Avian coronavirus/ Infectious bronchitis virus (IBV)	N	Hiscox et al. (2001)
Flaviviridae	n/a	Flavivirus	Dengue virus (DEN)	Core protein	Wang et al. (2002)
Flaviviridae	n/a	Flavivirus	Hepatitis C virus (HCV)	Core protein (wild-type and mutant), NS5B mutant	Realdon et al. (2004); Falcon et al. (2003); Hirano et al. (2003)

Flaviviridae	n/a	Flavivirus	Japanese encephalitis virus (JEV)	Core protein	Mori et al. (2005)
Flaviviridae	n/a	Flavivirus	West nile virus (WNV)	Core protein	Westaway et al. (1997)
Luteoviridae	n/a	Polerovirus	Potato leaf roll virus (PLRV)	CP, P5	Haupt et al. (2005)
Nodaviridae	n/a	Betanodavirus	Greasy grouper nervous necrosis virus (GGNNV)	Alpha	Guo et al. (2003)
Picornaviridae	n/a	Cardiovirus	Encephalomyocarditis virus (ECMV)	2a	Aminev et al. (2003a)
Potyviridae	n/a	Potyvirus	Potato virus A (PVA)	Nia	Rajamaki and Valkonen (2009)
Potyviridae	n/a	Potyvirus	Tabacco etch virus (TEV)	P3, NIa, Nib	Langenberg and Zhang (1997); Baunoch et al. (1991)
Potyviridae	n/a	Potyvirus	Turnip mosaic virus (TuMV)	VPg-Pro (Potential)	Beauchemin et al. (2007)
Togaviridae	n/a	Alphavirus	Semliki forest virus (SFV)	Core (C), nsP2	Michel et al. (1990); Rikkonen et al. (1992)
Baltimore class V: negative/ambisense, single-strand RNA					
Bornaviridae	n/a	Bornavirus	Borna disease virus (BDV)	Genome/anti-genome	Pyper et al. (1998)
Orthomyxoviridae	n/a	Influenzavirus A	Influenza A virus (IAV)	NS1, NP, M1, PB2	Davey et al. (1985); Emmott et al. (2010a)
Orthomyxoviridae	n/a	Isavirus	Infectious Salmon anaemia virus (ISAV)	Genome/anti-genome	Goic et al. (2008)
Paramyxoviridae	Paramyxovirinae	Avulavirus	Newcastle disease virus (NDV)	Matrix protein (M)	Peeples et al. (1992)
Rhabdoviridae	n/a	Nucleorhabdovirus	Maize fine streak virus (MFSV)	N, P	Tsai et al. (2005)

(continued)

Table 14.1 (continued)

Family	Subfamily	Genus	Virus	Protein/nucleic acid	Reference
Baltimore class VI: single-strand RNA – reverse transcription					
Retroviridae	Orthoretrovirinae	Betaretrovirus	Jaagsiekte sheep retrovirus (JSRV)	Env-SP/JSE-SP	Caporale et al. (2009)
Retroviridae	Orthoretrovirinae	Betaretrovirus	Mouse mammary tumour virus (MMTV)	Env-SP/p14, Rem	Hoch-Marchaim et al. (1998); Indik et al. (2005)
Retroviridae	Orthoretrovirinae	Deltaretrovirus	Human T cell lymphotropic virus 1 (HTLV-1)	HBZ-SP1, Rex, p21x (a natural Rex truncation), pX, p30, p30II, Unspliced viral mRNA	Hivin et al. (2007); Kubota et al. (1989); Kubota et al. (1996); Siomi et al. (1988); Ghorbel et al. (2006); Bartoe et al. (2000); Nosaka et al. (1989)
Retroviridae	Orthoretrovirinae	Deltaretrovirus	Human T cell lymphotropic virus 2 (HTLV-2)	Rex	Narayan et al. (2003)
Retroviridae	Orthoretrovirinae	Gammaretrovirus	Maloney murine leukaemia virus (MuLV/MMLV)	Nucleocapsid (NC), Integrase (IN)	Risco et al. (1995)
Retroviridae	Orthoretrovirinae	Lentivirus	Bovine immunodeficiency virus (BIV)	Tat, Rev	Gomez Corredor and Archambault (2009); Fong et al. (1997)
Retroviridae	Orthoretrovirinae	Lentivirus	Carpine arthritis encephalitis virus (CAEV)	Rev-C	Saltarelli et al. (1994)
Retroviridae	Orthoretrovirinae	Lentivirus	Human immunodeficiency virus 1 (HIV-1)	Tat, Rev, unspliced viral mRNA	Cochrane et al. (1990); Ruben et al. (1989)
Retroviridae	Orthoretrovirinae	Lentivirus	Human immunodeficiency virus 2 (HIV-2)	Tat, Rev	Orsini and Debouck (1996); Dillon et al. (1991)

Retroviridae	Orthoretrovirinae	Lentivirus	Visna virus	Rev-V	Schoberg and Clements (1994)
Baltimore class VII: double-strand DNA – reverse transcription					
Hepadnaviridae	n/a	Orthohepadnavirus	Hepatitis B virus (HBV)	Core (various mutants)	Ning and Shih (2004)
Subviral agents: satellite viruses – circular, negative-sense, single-strand RNA					
n/a	n/a	Deltavirus	Hepatitis D virus (HDV)	deltaAg (S and L), anti-genome, RNA	Macnaughton et al. (1990); Li et al. (2006); Kojima et al. (1989)
Subviral agents: Satellite viruses – linear, positive-sense, single-strand RNA					
n/a	n/a	n/a	Satellite panicum mosaic virus (SPMV)	CP	Qi et al. (2008)
Subviral agents: Viroids					
Pospiviroidae	n/a	Pospiviroid	Potato spindle tuber viroid	Genome (positive sense)	Harders et al. (1989)
Endogenous retroviruses					
n/a	n/a	n/a	Human endogenous retrovirus-K (HERV-K)	Env-SP, Np9, cORF	Ruggieri et al. (2009); Armbruester et al. (2004); Lower et al. (1995)

Many of these proteins have been shown to interact with nucleolar proteins

The reason why RNA viruses, and positive-strand RNA viruses in particular, interact with the nucleolus when the site of genome replication is in the cytoplasm is less intuitive. In this latter case, viral proteins that are normally required in the cytoplasm must transit through the nuclear pore complex both to and from the nucleus. This process is crucial for virus biology because if the viral proteins that are required for cytoplasmic functions such as RNA synthesis and encapsidation are sequestered in the nucleolus or nucleus, then progeny virus production will be affected as has been revealed by inhibitor and genetic studies (Lee et al. 2006; Tijms et al. 2002). Viruses may interact with the nucleolus to usurp host cell functions and recruit nucleolar proteins to facilitate virus replication. Investigating the interactions between viruses and the nucleolus may facilitate the design of novel anti-viral therapies both in terms of recombinant vaccines (Pei et al. 2008) and molecular intervention (Rossi et al. 2007), and also contribute to a more detailed understanding of the cell biology of the nucleolus.

For many years our understanding of the interaction of viruses and the nucleolus was phenomenological and focused on identifying viral proteins that localised to this structure, their mechanisms of trafficking and potential interaction with nucleolar proteins (e.g. see Table 14.1). However, recent research capitalising on advances in proteomics, viral genetics and cellular imaging techniques are beginning to increase our understanding of the mechanisms viruses use to subvert host cell nucleoli and facilitate virus biology (Hiscox et al. 2010).

New data are now emerging that support the view that many viruses interact with the nucleus and nucleolus, particularly to facilitate virus replication. One of the best-studied viruses in terms of viral interactions with the nucleolus is HIV-1 and is described in detail in Chap. 17. Although HIV has clearly defined cytoplasmic and nuclear replication strategies, the virus has a positive-sense RNA genome in the sense that the viral capsid contains two copies of positive-sense RNA, but these are reverse transcribed in the cytoplasm and then trafficked to the nucleus, where ultimately the new genome is transcribed and trafficked back to the cytoplasm. Part of the reasoning for the interaction of HIV-1 with the nucleolus is the trafficking of intronless mRNA from the nucleus into the cytoplasm (Michienzi et al. 2000). This is a property shared with herpes viruses and indicated that different viruses have evolved similar strategies involving subversion of nucleolar function for the benefit of virus biology (Boyne and Whitehouse 2006). In the case of HIV-1, this knowledge has also led to the design and implementation of effective genetic therapies against the virus (Unwalla et al. 2008).

14.2 DNA Virus Interactions with the Nucleolus

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. 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 (Salsman et al. 2008), 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, and are discussed later (Lam et al. 2010). Several different herpes virus proteins have been shown to cause the redistribution of nucleolar proteins and hence disruption of the nucleolus. These include herpes simplex virus 1, the major tegument structural protein VP22 (Lopez et al. 2008), and the US11 (Xing et al. 2010) and UL24 proteins (Bertrand and Pearson 2008; Lymberopoulos and Pearson 2007). Such disruption in many cases may have a direct effect on nucleolar function.

A significant area of virus biology that has been 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 (Dundr et al. 1995). Similar observations have also been made in herpesviruses (Boyne and Whitehouse 2006, 2009; Leenadevi and Dalziel 2009). Initial studies utilising the prototype γ-2 herpesvirus, herpes virus saimiri (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 (Boyne and Whitehouse 2006). Intriguingly, ablating ORF57 nucleolar trafficking led to a failure of ORF57-mediated viral mRNA nuclear export (Boyne and Whitehouse 2006). 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 (Boyne et al. 2008b). Moreover, on the rapid disorganisation of the nucleolus a reduction is observed in virus mRNA nuclear export (Boyne and Whitehouse 2009). 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 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 (Boyne et al. 2010). This interaction effectively enhances the translation of the predominantly intronless transcripts made by KSHV, and draws parallels with potential translation enhancement of positive strand RNA virus genomes through their interaction with the nucleolus (discussed later).

A second area of virus replication where nucleolar proteins are sequestered involves the replication of the virus DNA genome. For example, we (Matthews) and others have observed that nucleolar antigens upstream binding factor (UBF) and nucleophosmin (B23.1) are both sequestered into adenovirus DNA replication centres where they promote viral DNA replication (Hindley et al. 2007; Lawrence et al. 2006; Okuwaki et al. 2001). Similarly, in HSV-1 infected cells, a number of nucleolar proteins including nucleolin and UBF are recruited into viral DNA replication centres (Lymberopoulos and Pearson 2010). 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 acts in a dominant-negative manner inhibiting virus DNA replication (Stow et al. 2009). Moreover, depletion of nucleolin results in reduced virus gene expression and infectious virion production (Calle et al. 2008; Sagou et al. 2010).

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 homeodomain-interacting protein kinase 2 (HIPK2), which plays a role in p53-mediated cellular apoptosis and hypoxic response (Calzado et al. 2009) and also participates in the regulation of the cell cycle (Calzado et al. 2007). This interaction alters the sub-cellular localisation of HIPK2 and protects against HIPK2-mediated cell cycle arrest (Giraud et al. 2004). In contrast, the cellular protein, protein interacting with the carboxyl terminus-1 (PICT-1), can sequester the virally encoded apoptosis suppressor protein, KS-Bcl-2 protein, from the mitochondria into the nucleolus to down-regulate its anti-apoptotic activity (Kalt et al. 2010). This is a potential interesting interplay between two sub-cellular structures involved in the viral stress response (Olson 2009), and maybe more common and widespread. For example, bacterial infection has been shown to disrupt the nucleolus through regulating mitochondrial dysfunction (Dean et al. 2010).

14.3 Interactions of RNA Viruses with the Nucleolus

Although many RNA virus proteins have been shown to localise to the nucleolus, most attention has focused on viral capsid proteins. These are proteins that associate with the viral genome for encapsidation and assembly of new virus particles. These proteins may also modulate replication (and transcription, where appropriate) of the viral genome. Increasingly, capsid proteins have also been shown to have a number of roles in modulating host cell signalling pathways and functions. These capsid proteins are referred to as capsid, nucleoproteins or nucleocapsid proteins, depending on the virus. In many cases, they are phosphorylated (Chen et al. 2005), which can modulate activity (Spencer et al. 2008).

Many examples of these proteins have been shown to localise to the nucleolus both when over-expressed and also in infected cells. These include proteins from positive-strand animal and plant RNA viruses, including the coronavirus nucleocapsid protein (Chen et al. 2002; Hiscox et al. 2001; Wurm et al. 2001), the arterivirus nucleocapsid protein (Rowland et al. 1999), the alphavirus capsid protein (Jakob 1994) and non-structural protein nsP2 (Rikkonen et al. 1992, 1994) and the umbravirus ORF3 protein (Ryabov et al. 2004). Capsid proteins from negative-strand RNA viruses also localise to the nucleolus. These have strain dependent localisation of a number of different influenza virus proteins (Emmott et al. 2010c; Han et al. 2010; Melen et al. 2007; Volmer et al. 2010).

For many years this has followed a phenomenological pattern and viral capsid and RNA-binding proteins might simply localise to the nucleolus because they diffuse through the nuclear pore complex and associate with compartments in the nucleus that have high RNA contents – the nucleolus in particular because it is transcriptionally active. In this case, sub-cellular localisation to the nucleolus would have no physiological consequence for the virus or the cell. However, RNA virus replication is error prone and selection pressure might apply to such a fortuitous localisation (given the ~4,500+ nucleolar proteins and their diverse roles (Ahmad et al. 2009)), with the concomitant effect that the virus could select for changes that ultimately disrupt nucleolar function and/or recruit nucleolar proteins to aid virus replication.

There is a potential correlation between the nucleolar localisation of a viral protein and the loss of an essential nucleolar function. The molecular mechanisms responsible for this effect are unknown, but the displacement and re-localisation of nucleolar proteins by viral proteins could increase or decrease the nucleolar, nuclear and/or cytoplasmic pool of these proteins. Certainly, the accumulation of viral proteins in the nucleolus could potentially cause volume exclusion or crowding effects, which have been proposed to play a fundamental role in the formation of nuclear compartments including the nucleolus, and can be addressed by proteomic strategies. Therefore, disruption of nucleolar architecture and function might be common in virus-infected cells if viral proteins target the nucleolus or a stage of the virus lifecycle disrupts nucleolar proteins. For example, poliovirus infection results in the selective redistribution of nucleolin from the nucleolus to the cytoplasm (Waggoner and Sarnow 1998) and inactivation of UBF, which shuts off RNA polymerase I transcription in the host cell. The infection of cells with IBV has been shown to disrupt nucleolar architecture (Dove et al. 2006b) and cause arrest of the cell cycle in the G2/M phase and failure of cytokinesis (Dove et al. 2006a). The IBV and arterivirus nucleocapsid proteins associate with nucleolin and fibrillarin, respectively. Similarly, the HIV-1 Rev protein has been shown to localise to the DFC and GC and over-expression of Rev protein alters the nucleolar architecture and is associated with the accumulation of nucleophosmin (Dundr et al. 1995).

14.4 Trafficking of Virus Proteins to the Nucleolus

Many different virus proteins localise to the nucleolus (Table 14.1). However, predicting viral (and cellular) nucleolar targeting signals has historically been problematic and only recently has bioinformatic software been developed to fascilitate this (Scott et al. 2011). Nucleolar trafficking might be mediated by virtue of the fact that viral proteins that are trafficked to the nucleolus contain motifs that resemble host nucleolar targeting signals, that is, a form of molecular mimicry is used (Rowland and Yoo 2003). The discovery of specific nucleolar trafficking signals in viral proteins has indicated a functional mechanism behind this observed localisation (Lee et al. 2003; Reed et al. 2006; Rowland et al. 2003). Analysis of the

different nucleolar trafficking signals identified in viral proteins using dynamic live-cell imaging has certainly demonstrated that different proteins can confer differential trafficking rates and localisation patterns (Emmott et al. 2008). This is very similar to cellular nucleolar proteins (Lechertier et al. 2007).

In some virus proteins, both NLSs and nucleolar targeting signals act in concert to direct a protein to the nucleolus. The arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) nucleocapsid protein localises to the nucleolus and has been shown to contain two potential NLSs, a pat4 and a downstream pat7 motif (Rowland et al. 1999, 2003). Analysis revealed that a 31 amino acid sequence incorporating the pat7 motif could direct the nucleocapsid protein to both the nucleus and nucleolus. The protein also contains a predicted NES, presumably to allow the protein to traffic back into the cytoplasm to contribute to viral function in this compartment. This is common with other similar related proteins. For example, in the avian coronavirus nucleocapsid protein an eight amino acid sequence is necessary and sufficient to target the protein to the nucleolus (Reed et al. 2006) and contains an NES (Reed et al. 2007). Intriguingly, genetic analysis (Lee et al. 2006), dynamic live-cell imaging (You et al. 2008) and use of trafficking inhibitors (Tijms et al. 2002) paint a picture of the requirement of these positive sense RNA virus capsid proteins localising to the nucleolus as soon as they are translated, prior to their involvement in virus replication or assembly. This may be related to subversion of host cell function, protein modification (e.g. phosphorylation) or recruitment of nucleolar proteins.

Viral proteins might also traffic to the nucleolus through association with cellular nucleolar proteins (Yoo et al. 2003). For example, the hepatitis delta antigen has been shown to contain a nucleolar targeting signal that also corresponded to a site that promoted binding to nucleolin (Lee et al. 1998). Mutating this region prevented nucleolin binding to the delta antigen and nucleolar trafficking. By implication, this relates nucleolin binding to nucleolar trafficking (Lee et al. 1998). Certainly, interaction with nucleophosmin and hepatitis delta antigens can modulate viral replication (Huang et al. 2001) and more recently combined proteomic-RNAi screens have revealed many other nucleolar proteins that can be associated with this viral protein (Cao et al. 2009). Trafficking and accumulation of viral proteins to and from the nucleolus, similar to cellular proteins, may also be cell cycle related. For example, the coronavirus nucleocapsid protein localises preferentially to the nucleolus in the G2 phase of the cell cycle (Cawood et al. 2007), as does the human cytomegalovirus protein UL83 in the G1 phase (Arcangeletti et al. 2011). Again these trafficking profiles may be related to the interaction with cellular nucleolar proteins (Emmott and Hiscox 2009).

14.5 Functional Relevance of Nucleolar Interactions to the Viral Life Cycle

Many different examples now exist to show that the disruption of nuclear or nucleolar trafficking of viral proteins affects viral pathogenesis, and argues against nucleolar localisation as a purely phenomenological observation. For example, the

Semliki Forest virus non-structural protein nsP2 can localise to the nucleolus (Peranen et al. 1990; Rikkonen et al. 1992, 1994) and disruption of this localisation through a single amino acid change results in a reduction in neurovirulence (Fazakerley et al. 2002). Such in vitro data has also been backed up by persuasive in vivo data. Mutation of the arterivirus nucleocapsid protein pat7 NLS motif in the context of a full-length clone revealed that this sequence could have a key role in virus pathogenesis in vivo, as animals infected with mutant viruses had shorter viraemia than wild-type viruses (Lee et al. 2006; Pei et al. 2008). Interestingly, reversions occurred in the mutated nucleocapsid gene sequence and although the amino acid sequence of the pat7 motif was altered, its function was not; this new signal was defined as a pat8 motif (Lee et al. 2006). The clear implications of this groundbreaking work is that disruption of nucleolar trafficking of a viral protein proves functional relevance and illustrates the potential of exploiting this knowledge for the generation of growth attenuated recombinant vaccines (Pei et al. 2008; Reed et al. 2006, 2007).

Similarly, point mutations in the Japanese encephalitis virus (JEV) core protein that abolished nuclear and nucleolar localisation resulted in recombinant viruses with impaired replication in mammalian cells, compared to wild type virus (Mori et al. 2005; Tsuda et al. 2006). Curiously, replication of recombinant viruses was not impaired in insect cells, illustrating this could potentially be related to differences in nucleolar architecture and proteomes between these cell types (Thiry and Lafontaine 2005). The JEV core protein has been shown to interact with nucleophosmin and is translocated from the nucleolus to the cytoplasm.

Flaviviruses in general (JEV, Dengue virus and West Nile virus) appear to have a part-nuclear stage to the synthesis of viral RNA and several components of the viral replicase together with newly synthesised RNA have been found in the nucleus of infected cells (Uchil et al. 2006). One intriguing question that has yet to be elucidated is how such viral RNA traffics from the nucleus to the cytoplasm. Most cellular mRNAs are spliced and it is part of the splicing process that signals nuclear export. Certain DNA viruses, such as herpesvirus saimiri, produce intron-less mRNA and these viruses have evolved specific viral proteins (such as herpesvirus saimiri ORF57 (Boyne et al. 2008a)), which interact with the cellular mRNA export machinery (e.g. the mRNA processing and export factor ALY) to traffic viral mRNA from the nucleus to the cytoplasm (Boyne et al. 2008b, 2010; Boyne and Whitehouse 2006) and a similar process might be required by RNA viruses. For example, tomato bushy stunt virus (TBSV) redistributes ALY from the nucleus to the cytoplasm, and this might be a way the virus mediates host cell protein synthesis (Uhrig et al. 2004). In plants RNA silencing, a host defence mechanism targets virus RNAs for degradation in a sequence-specific manner and viruses use several mechanisms to counteract this system (Canto et al. 2006). TBSV encodes a protein, P19, which interferes with this pathway. However, ALY might transport P19 from the cytoplasm to the nucleus or nucleolus and disrupt its silencing suppression activity. Nucleolin has also been shown to be involved in the trafficking of herpes simplex virus type 1 nucleocapsids from the nucleus to the cytoplasm (Sagou et al. 2010), drawing parallels with the involvement of nucleolar proteins in the movement of plant viruses

(Kim et al. 2007a, b). Different plant virus proteins involved in long-distance phloem-associated movement of virus particles or with roles in binding to the RNA virus genomes localise to the nucleolus and other sub-nuclear structures (Kim et al. 2007b; Ryabov et al. 2004). This may be mediated by association with nuclear proteins, as is the case with fibrillarin and the ORF3 protein of plant umbraviruses (Kim et al. 2007a).

Hijacking the nucleolus is not exclusive to plant viruses and may also occur with mammalian viruses. Similar to the plant rhabdovirus maize fine streak virus (MFSV), whose nucleocapsid and phosphoproteins localise to the nucleolus (Tsai et al. 2005), the animal negative-stranded RNA virus Borna disease virus has been reported to use the nucleolus as a site for genome replication, and its RNA-binding protein has the appropriate trafficking signals for import to and export from the cytoplasm to the nucleus (Pyper et al. 1998). The hepatitis delta virus genome also has differential synthesis in the nucleus with RNA being transcribed in the nucleolus (Huang et al. 2001); this is similar to the potato spindle tuber viroid where RNAs of opposite polarity are sequestered in different nuclear compartments, with the positive-sense RNA being transported to the nucleolus. Again localisation to different sub-nuclear strcutures may have different roles in the virus life cycle (Li et al. 2006). An intriguing recent discovery has been made showing that adeno-associated virus (AAV) encodes an additional protein called assembly-activating protein (AAP) that localises to the nucleolus and promotes assembly of the viral capsid (Sonntag et al. 2010).

As a result of their limited genomes and coding capacities, recruitment of cellular proteins with defined functions in RNA metabolism would be a logical step to facilitate RNA virus infection. As nucleolar proteins have many crucial functions in cellular RNA biosynthesis, processing and translation, it comes as no surprise that nucleolar proteins are incorporated into the replication and/or translation complexes formed by RNA viruses. Given that some nucleolar proteins have many different functions, the same nucleolar protein might be used by a virus for different aspects of the replication pathway. Studies suggest that the human rhinovirus 3 C protease (3Cpro) pre-cursors, 3CD' and/or 3CD, localise in the nucleoli of infected cells early in infection and inhibit cellular RNA transcription via proteolytic mechanisms (Amineva et al. 2004). This general property is not restricted to human rhinovirus and in terms of the inhibition of cellular translation has also been described for encephalomyocarditis virus (Aminev et al. 2003a, b), again suggesting roles in translational regulation.

14.6 Applying Quantitative Proteomics to Study Viral Interactions with the Nucleolar Proteome

Given the many roles of the nucleolus in the life cycle of the cell, including as stress sensor (Boulon et al. 2010; Mayer and Grummt 2005), it would seem reasonable that comprehensive unbiased analysis of the nucleolar proteome would yield interesting data, particularly, with providing clues as to what cellular nucleolar functions may

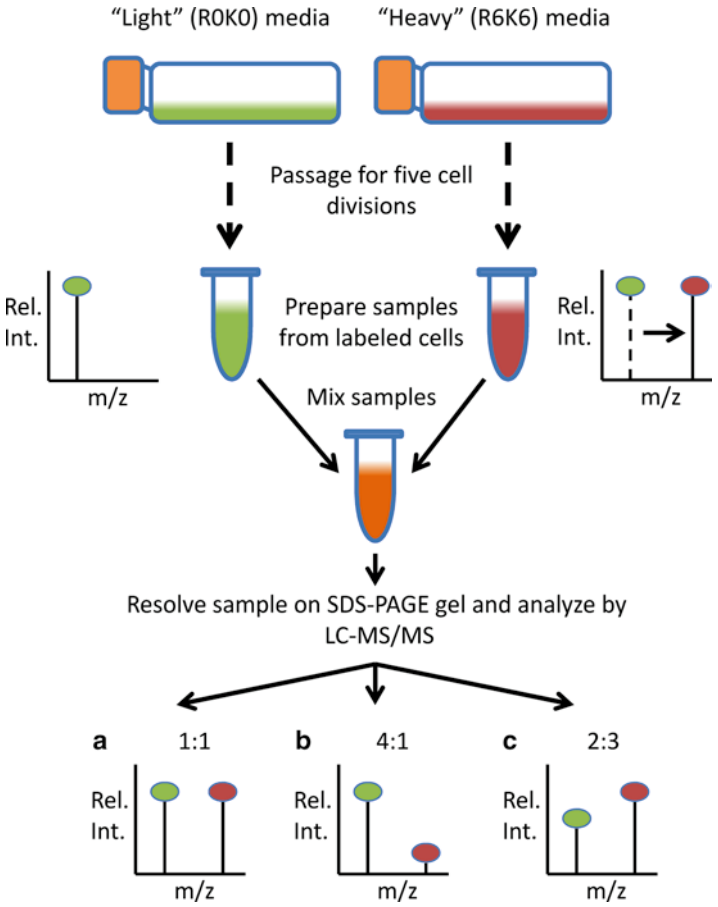

Fig. 14.1 Diagram of a "classic" SILAC experiment. This technology allows high-throughput quantitative proteomics and has been readily applied to the nucleolus, especially when coupled with dynamic live-cell imaging (Andersen et al. 2005). The ability to simultaneously compare up to three different conditions through selection of the appropriate isotope label has enabled the recent studies of how the nucleolar proteome changes in virus-infected cells (Emmott et al. 2010a; Emmott et al. 2010b; Emmott et al. 2010c; Hiscox et al. 2010; Lam et al. 2010)

be altered by virus infection and what mechanisms the nucleolus may use to respond to this. How the nucleolar proteome changes in response to virus-infection has been investigated using stable isotope labelling with amino acids in cell culture (SILAC) coupled to LC-MS/MS and bioinformatics (Fig. 14.1). These studies, led by our laboratories, have analysed purified nucleoli and the nucleus, and have directly stemmed from the pioneering work of the Lamond laboratory in analysing purified nucleoli using quantitative proteomics (Andersen et al. 2005). Viruses investigated so far have included human adenovirus (Lam et al. 2010), avian coronavirus (Emmott et al. 2010a, b), different strains of influenza virus (Emmott et al. 2010c) and human respiratory syncytial virus (Munday et al. 2010). Overall, our data indicates that only a small proportion of nucleolar proteins change in abundance in virus-infected cells,

and these tend to be virus-specific. For example, in adenovirus infected cells just 7% of proteins identified show a twofold or greater change compared to almost a third of nucleolar antigens showing a greater than twofold change when cells are treated with ActD which inhibits rRNA synthesis (Lam et al. 2010). What is notable is that direct comparison between the adenovirus data set and the ActD dataset shows no clear correlation (Hiscox et al. 2010; Lam et al. 2010), 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 a previous observation that adenovirus infection does not affect rRNA synthesis even 36 h post-infection (Lawrence et al. 2006). These results were initially surprising given the number of different viral proteins that can localise to this structure and how they interact with nucleolar proteins. This suggests that the nucleolar proteome and architecture is resilient during early stages of infection but may become disrupted as more and more damage accumulates inside cells because of virus activity, as clearly evidenced in live-cell imaging experiments (Bertrand and Pearson 2008; Dove et al. 2006b; Lymberopoulos et al. 2010).

14.7 Future Research Directions

Coupling quantitative proteomic analysis of the nucleolus and deep sequencing throughout infection in time-course experiments of lytic, latent, acute and persistent viruses would reveal valuable insights into the response of the nucleolus to virus infection. Likewise, being able to move from studying cell culture-adapted laboratory strains into clinical isolates replicating in primary cells would yield more biologically relevant information, particularly with regard to the severity of disease and nucleolar changes. These technologies could also be applied to large-scale analysis of viral proteins that traffic to the nucleolus and the cellular nucleolar proteins that they associate with (e.g. using SILAC and EGFP-traps (Trinkle-Mulcahy et al. 2008)), thus generating and integrating interactome networks with the nucleolar proteome during infection.

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