Intracellular Trafficking of Retroviral Gag and RNA
During Late Replication

A Dissertation in
Cell and Molecular Biology
by
Darrin V. Bann

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The dissertation of Darrin V. Bann was reviewed and approved* by the following:

Leslie J. Parent  
Professor of Medicine and Microbiology & Immunology  
Chief, Division of Infectious Diseases  
Dissertation Advisor  
Chair of Committee

Sarah K. Bronson  
Associate Professor of Cellular & Molecular Physiology  
Director, Cellular & Molecular Biology Graduate Program

Edward J. Gunther  
Associate Professor of Medicine

Jeffery T. Sample  
Professor of Microbiology & Immunology

David J. Spector  
Professor and Distinguished Educator of Microbiology & Immunology

*Signatures are on file in the Graduate School.
Abstract

Retroelements, including retroviruses and retrotransposons, are ubiquitous throughout eukaryotes and replicate by reverse-transcribing their RNA genome into DNA and stably integrating into the chromosome of the host cell. Retroviruses encode a very limited genome and the most simple retroviruses carry out all of the functions needed to replicate using only three proteins: Gag, the viral structural protein that orchestrates the assembly of nascent virus particles using the genomic RNA as a scaffold; Pol, the reverse transcriptase and integrase; and Env, the envelope glycoprotein that studs the outside of virus particles. Because retroviruses encode such a limited genome, they are dependent on host factors to mediate or facilitate many aspects of the virus life cycle including transcription of viral RNAs, assembly of nascent particles, and targeting viral proteins to the plasma membrane where budding occurs. However, the host factors involved in many aspects of retrovirus replication are poorly characterized. Our goal was to understand the intracellular trafficking pathways of retroviral Gag and RNA during the late (post-integration) phase of replication to identify cellular factors implicated in genome encapsidation, particle assembly, and plasma membrane localization of Gag.

After integration, full-length retroviral RNA is transcribed by cellular machinery and exported from the nucleus. This full-length RNA serves two roles in retroviral replication, acting as an mRNA to direct the translation of the Gag, Gag-Pro, and Gag-Pro-Pol structural proteins, and as a genomic RNA (gRNA), which is encapsidated into nascent virus particles. The requirement for
retroviruses to efficiently assemble virus particles suggests that a mechanism exists to separate full-length RNA to be used for translation from identical RNA molecules destined for encapsidation. However, the mechanisms that retroviruses use to separate RNA molecules destined for these two fates have remained an enigma. We hypothesized that cellular RNA-binding proteins interact with the viral RNA and/or Gag to target a population of viral RNA for encapsidation.

To test this hypothesis, we chose mouse mammary tumor virus (MMTV) as a model system. MMTV assembles complete, immature capsids in the cytoplasm, so we reasoned that sites of capsid assembly might also be the location where Gag first interacts with the viral genome. Therefore, by studying cellular factors associated with these sites of assembly, we could gain insight into cellular proteins that target viral RNA for encapsidation. As part of an unbiased screen to identify cellular proteins that interact with MMTV Gag, we identified ribosomal protein, large 9 (RPL9) as a Gag-interacting partner. A single RPL9 protein is present in each large ribosomal subunit and, strikingly, overexpression of RPL9 induced the accumulation of Gag and RPL9 in nucleoli where ribosome subunit assembly occurs. Furthermore, Gag interacted with RPL9 in nucleoli and under steady state conditions we identified a population of Gag within nucleoli. Depletion of extraribosomal RPL9 using siRNA resulted in decreased MMTV virus production, suggesting that Gag interacts with RPL9 in the nucleolus to facilitate virus production. Although the mechanism by which RPL9 influences virus production is not known, because RPL9 is an RNA-binding protein one
possibility is that RPL9 interacts with Gag to help Gag identify viral genomes for encapsidation.

Several other cellular proteins have also been reported to facilitate encapsidation of viral RNAs and assembly of virus particles. For example, the Gag protein and genomic RNA of the yeast Ty3 retrotransposon, which follows an assembly pathway similar to MMTV, interact with a conserved set of cellular mRNA-binding proteins associated with stress granules (SGs) and processing bodies (PBs) to facilitate Ty3 replication. These observations led us to conduct parallel experiments in MMTV to test whether SG and PB proteins also influenced MMTV particle assembly. We found that specific SG- and PB-associated proteins colocalized with MMTV Gag in the cytoplasm in translationally-repressed complexes. Furthermore, a subset of these proteins interacted with MMTV Gag through an RNA-dependent mechanism and colocalized with a subviral RNA reporter. To our surprise, the viral RNA appeared to recruit SG-associated proteins and Gag into complexes, suggesting that SG and/or PB proteins may bind the viral RNA and serve as a “landmark” used by Gag to identify viral RNAs for encapsidation. Indeed, reducing the expression of specific SG or PB-associated proteins using siRNAs resulted in decreased virus production, indicating that SG- and PB-associated proteins play an essential role in efficient virus production. Together, these data led to a model where SG- and/or PB-associated proteins repress the translation of a portion of viral RNA, thereby targeting this RNA for encapsidation.
After selecting a genome for encapsidation all retroviral Gag proteins must traffic to the plasma membrane to bud from the host cell as a virus particle. Gag proteins of many retroviruses recognize the plasma membrane by the presence of the plasma membrane-specific phospholipid phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂]. The Gag protein of Rous sarcoma virus (RSV) transiently traffics through the nucleus and then, in contrast to MMTV, localizes predominantly to the plasma membrane, which serves as the site of virus particle assembly. However, previous reports indicated that RSV Gag localized to the plasma membrane through a PI(4,5)P₂-independent mechanism. By contrast, we found that enzymatic depletion of PI(4,5)P₂ caused the mis-localization of RSV Gag from the plasma membrane to an intracellular compartment. Strikingly, we found that mutants of RSV Gag deficient in nuclear trafficking were also less sensitive to the depletion of PI(4,5)P₂, suggesting a previously unknown role of nuclear membrane-free PI(4,5)P₂ in RSV replication. Together, these data shed light on novel ways in which retroviruses interact with cellular proteins to locate the viral genome, assemble virus particles, and achieve plasma membrane localization required for budding.
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</thead>
<tbody>
<tr>
<td>Ago</td>
<td>Argonaute-family protein</td>
</tr>
<tr>
<td>ALV</td>
<td>Avian leucosis virus</td>
</tr>
<tr>
<td>APOBEC</td>
<td>Apolipoprotein mRNA editing enzyme, catalytic</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>ARM</td>
<td>Arginine rich motif</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid</td>
</tr>
<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
</tr>
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<td>ColIP</td>
<td>Co-immunoprecipitation</td>
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<td>Crm1</td>
<td>Chromosome region maintenance-1</td>
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<td>CSD</td>
<td>Cold shock domain</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CTE</td>
<td>Constitutive transport element</td>
</tr>
<tr>
<td>CTRS</td>
<td>Cytoplasmic targeting/retention signal</td>
</tr>
<tr>
<td>DFC</td>
<td>Dense fibrillar component</td>
</tr>
<tr>
<td>DIS</td>
<td>Dimerization initiation site</td>
</tr>
<tr>
<td>DLS</td>
<td>Dimerization linkage structure</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>Direct repeat</td>
</tr>
<tr>
<td>EIAV</td>
<td>Equine infectious anemia virus</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic Initiation Factor 2 α</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAST</td>
<td>Fas-activated serine/threonine phosphoprotein</td>
</tr>
<tr>
<td>FC</td>
<td>Fibrillar center</td>
</tr>
<tr>
<td>Fig</td>
<td>Figure</td>
</tr>
<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<tr>
<td>G3BP1</td>
<td>Ras GTPase activating-protein (SH3 domain) binding protein-1</td>
</tr>
<tr>
<td>Gag</td>
<td>Group-specific Antigen</td>
</tr>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
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<td>GC</td>
<td>Granular component</td>
</tr>
<tr>
<td>GCN-2</td>
<td>General Control Nonderepressable-2</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage-colony stimulating factor</td>
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<tr>
<td>gRNA</td>
<td>Genomic RNA</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type-1</td>
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<tr>
<td>HIV-2</td>
<td>Human immunodeficiency virus type-2</td>
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<tr>
<td>HRE</td>
<td>Hormone responsive element</td>
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<tr>
<td>HRI</td>
<td>Heme-regulated Inhibitor</td>
</tr>
<tr>
<td>HS</td>
<td>Heat shock</td>
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<td>HTLV-1</td>
<td>Human T-cell leukemia virus type-1</td>
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<td>HuR</td>
<td>Human antigen R</td>
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<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
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<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>JSRV</td>
<td>Jaaagsiekte sheep retrovirus</td>
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<td>L9</td>
<td>Ribosomal protein, large 9</td>
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<td>LEDGF</td>
<td>Lens epithelium-derived growth factor</td>
</tr>
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<td>LINE-1</td>
<td>Long interspersed nuclear element-1</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
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<tr>
<td>M-PMV</td>
<td>Mason-Pfizer monkey virus</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
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<tr>
<td>miRNA</td>
<td>Micro-RNA</td>
</tr>
<tr>
<td>MLV</td>
<td>Murine leukemia virus</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nMDP</td>
<td>Normalized mean deviation product</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NMuMG</td>
<td>Normal murine mammary gland epithelial cells</td>
</tr>
<tr>
<td>NoLS</td>
<td>Nucleolar localization signal</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
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<td>P-body, PB</td>
<td>Processing Body</td>
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<td>PABP1</td>
<td>Poly(A)-binding protein-1</td>
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<td>PERK</td>
<td>Pancreatic Endoplasmic Reticulum eIF2α Kinase</td>
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<tr>
<td>PFV-1</td>
<td>Primate foamy virus-1</td>
</tr>
<tr>
<td>PI(4,5)P₂</td>
<td>Phosphatidylinositol-(4,5)-bisphosphate</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein Kinase R</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>pp21</td>
<td>Phosphoprotein 21</td>
</tr>
<tr>
<td>PPT</td>
<td>Polypyrimidine tract</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>Pro</td>
<td>Protease</td>
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<tr>
<td>Rem</td>
<td>Regulator of export of MMTV mRNAs</td>
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<td>RemRE</td>
<td>Rem response element</td>
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<td>RHA</td>
<td>RNA helicase A</td>
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<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNA</td>
<td>Ribose nucleic acid</td>
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<td>RNP</td>
<td>Ribonucleoprotein complex</td>
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<td>Ribosomal protein, large 11</td>
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<td>Ribosomal protein, small 6</td>
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<td>RPS7</td>
<td>Ribosomal protein, small 7</td>
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<td>RRE</td>
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<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td>RSV</td>
<td>Rous sarcoma virus</td>
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<tr>
<td>RT</td>
<td>Reverse transcription</td>
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<tr>
<td>SA</td>
<td>Splice acceptor</td>
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<tr>
<td>Sag</td>
<td>Superantigen</td>
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<tr>
<td>SD</td>
<td>Splice donor</td>
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<tr>
<td>SG</td>
<td>Stress granule</td>
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<tr>
<td>SHRNP</td>
<td>Staufen-1 HIV-1-dependent ribonucleoprotein complex</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SL</td>
<td>Stem-loop</td>
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<td>SP</td>
<td>Spacer peptide of RSV Gag; Signal peptide of MMTV Rem</td>
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<td>SU</td>
<td>Surface domain of Env</td>
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<td>svRNA</td>
<td>Sub viral RNA</td>
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<td>Trans Golgi network 38</td>
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<td>TIA1</td>
<td>T-cell intracellular antigen cytotoxic granule-associated RNA-binding protein-1</td>
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<td>TIAR</td>
<td>TIA1 cytotoxic granule-associated RNA binding protein-like-1</td>
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<td>TM</td>
<td>Transmembrane domain of Env</td>
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<td>tRNA</td>
<td>Transfer RNA</td>
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<td>TTP</td>
<td>Tristetraprolin</td>
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<td>UBF</td>
<td>Upstream binding factor</td>
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<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>VLP</td>
<td>Virus-like particle</td>
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<td>YB1</td>
<td>Y-box binding protein 1</td>
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<td>ZBP1</td>
<td>Zipcode binding protein-1</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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</table>
Preface

For the work described in Chapter 2 (Appendix A) the following individuals conducted the experiments:

| Figure 1 | Ingrid S. Pultz, Stephen P. Goff, and Tatyana V. Golovkina |
| Figure 2A | Darrin V. Bann |
| Figure 2B | Melissa Kane |
| Figure 2C | Darrin V. Bann |
| Figure 2D | Andrea R. Beyer |
| Figure 3 | Andrea R. Beyer |
| Figure 4A | Darrin V. Bann |
| Figure 4B-D | Andrea R. Beyer |
| Figures 5 – 7 | Andrea R. Beyer |
| Figure 8 | Darrin V. Bann |

Darrin V. Bann and Andrea R. Beyer conducted statistical analysis of the data and wrote the manuscript with Leslie J. Parent.

For the work described in Chapter 6 (Appendix B) the following individuals conducted the experiments:

| Figure 1 | Darrin V. Bann and Shorena Nadaraia-Hoke |
| Figure 2A | Darrin V. Bann and Shorena Nadaraia-Hoke |
| Figure 2B | Timothy L. Lochmann and Nicole Gudleski-O'Regan |
| Figure 3 | Darrin V. Bann and Shorena Nadaraia-Hoke |
| Figure 4 | Darrin V. Bann and Shorena Nadaraia-Hoke |
| Figure 5 | Darrin V. Bann and Shorena Nadaraia-Hoke |

Darrin V. Bann conducted statistical analysis of the data, and Darrin V. Bann, Shorena Nadaraia-Hoke, and Leslie J. Parent wrote the manuscript.
Chapter 1

Literature Review
1.1 Introduction

Retroviruses are ubiquitous among Eukaryotes and are an important cause of cancer and immunodeficiency in animals. However, because retroviruses stably integrate into the genome of the host organism they also play a major role in evolutionary change. Despite the importance of retroviruses in human health and disease and the fact that the first animal retroviruses were described over 100 years ago, we are still working to understand basic mechanisms underlying retroviral replication and how retroviruses manipulate the cellular environment.

The first retrovirus, now called avian leucosis virus (ALV), was discovered by Ellermann and Bang in 1908 as a cause of leukemia and lymphoma in chickens [1]. Shortly thereafter, Peyton Rous discovered in 1911 that a form of transmissible sarcoma in chickens was actually caused by a filterable agent [2,3]. The virus, which now bears his name, Rous sarcoma virus (RSV), was the first viral cause of solid tumors to be discovered, for which Peyton Rous was awarded the Nobel Prize in Physiology or Medicine in 1966. The first virus to cause solid tumors in mammals, now called mouse mammary tumor virus (MMTV), was subsequently discovered in 1936 by John Bittner, who found that mammary cancer in mice could be transmitted from mothers to their offspring by a filterable agent in milk [4].

Through the past century of research, many molecular details of retrovirus replication are now well-understood, however each new discovery invariably raises more questions. Perhaps one of the most fascinating questions in retrovirology is: how do retroviruses carry out every function required for
replication using only a limited genome? Indeed, the most simple of retroviruses encodes only three genes: *gag*, *pol*, and *env*. As a result, each gene product may play several roles in the virus replication cycle. Moreover, like all viruses, retroviruses are intracellular parasites and have evolved to “hijack” cellular machinery to mediate their own replication. In turn, however, cells have evolved a number of defensive pathways designed to detect and inhibit viral replication, which retroviruses must disable or avoid using only a limited number of viral proteins. Accordingly, retroviral replication can be viewed as an intracellular battle between viral factors trying to promote viral replication, and cellular factors working to inhibit virus replication. However, the host factors implicated in retrovirus replication are only beginning to be understood.

The work within this dissertation is focused on describing interactions between cellular factors and retroviral Gag proteins that facilitate virus assembly. For instance, we found that a portion of MMTV Gag traffics through the nucleolus, where it interacts with a ribosomal protein called RPL9. This interaction likely occurs outside the context of the ribosome and is essential for efficient MMTV replication, suggesting that Gag may use RPL9 to carry out a previously uncharacterized extraribosomal function. Furthermore, we found that MMTV hijacks cellular RNA-binding proteins involved in antiviral defense to facilitate its own replication, suggesting that MMTV has evolved mechanisms to circumvent this defensive pathway. Finally, using RSV we discovered a potential link between nuclear trafficking of Gag and the mechanism that Gag uses to target the plasma membrane. As a result, this work identifies novel ways in which
retroviruses interact with host factors and manipulate the cellular environment to facilitate their own replication.

1.2 Classification of Retroviruses

Retroviruses are members of the family *Retroviridae* and are enveloped viruses containing a dimeric, positive-stranded RNA genome. The *Retroviridae* are unique among RNA viruses in that their RNA is reverse-transcribed to DNA before integrating into the cellular genome [5,6]. Most extant retroviruses are transmitted between individual organisms as exogenous viruses, however, if an exogenous retrovirus integrates into the genome of a germ cell it can become a permanent part of that organism’s genome as an endogenous retrovirus. Amazingly, almost 8.3% of the human genome is derived from endogenous retroviruses [7], indicating that retroviruses may be a driving force in human evolution.

Phylogenetic analysis of *Retroviridae* based on the protein sequence of the polymerase protein (Pol) revealed three major clades composed of seven distinct genera [8,9]. The first clade (class 1) encompasses the *Gammaretrovirus* and *Epsilonretrovirus* genera; the second (class 2) *Lentivirus, Deltaretrovirus, Alpharetrovirus, and Betaretrovirus*; and the third (class 3) *Spumaretrovirus* [9]. Classes 1 and 2 comprise the *Orthoretroviridae* or “true retrovirus” subfamily, while class 3 is comprised entirely of the *Spumaretrovirus* subfamily. All pathogenic retroviruses fall into classes 1 and 2, as the spumaviruses (or foamy viruses) cause no known pathology in animal hosts [10]. The focus of this
dissertation will fall on Rous sarcoma virus (RSV), an *Alpharetrovirus* that causes sarcoma in domesticated fowl, and mouse mammary tumor virus (MMTV), a *Betaretrovirus* that causes mammary tumors in mice.

In addition to being characterized by sequence similarity and pathogenicity, retroviruses have also historically been characterized by morphology. Type C retroviruses, such as RSV, assemble particles at the plasma membrane in a process that is concurrent with budding, and the mature virions have a round or angular centrally placed core. In contrast B-type viruses like MMTV assemble complete immature capsids in the cytoplasm, and the mature virus particles have round, eccentrically placed cores. Like B-type viruses, type D viruses assemble in the cytoplasm, however the mature particles have bar-shaped cores. Finally, the A-type morphology was originally used to describe the cytoplasmic precursor particles of B- and D-type viruses, however this term is now used in reference to intracisternal type-A particles, which are produced by an endogenous retrovirus and bud into the endoplasmic reticulum [1].

1.3 Characteristics of Retroviruses

1.3.1 General Features of MMTV and RSV Particles

Retrovirus particles are generally 80 – 120 nm in size and are composed of a lipid envelope, derived from the plasma membrane of an infected cell, surrounding a core of viral protein and RNA (Fig. 1.1A,B) [11]. For MMTV,
Figure 1.1 Immature vs. mature virus particles and the Gag proteins of MMTV and RSV. (A) A cartoon depicting immature and (B) mature retrovirus particles. (C) A map of the MMTV Gag protein. The squiggly line attached to MA indicates myristoylation of MMTV Gag. Numbers above the protein indicate amino acid positions, while arrows below the protein indicate protease cleavage sites. (D) A map of the RSV Gag protein, annotated as in (C). The colors of the MA, CA, and NC domains are the same for all parts of the figure. Domains specific to each Gag protein are colored white.
cryoelectron microscopy studies suggest an average particle diameter ranging from 130 – 140 nm [12], however the estimated size of virus particles seems to be dependent on sample preparation, as historical studies of MMTV report particle sizes ranging from 105 nm to 180 nm [13,14]. As a point of comparison, RSV particles also range in size from about 110 – 140 nm [15]. Although the size of MMTV cores is not related to the size of virus particles, consistent with the cytoplasmic assembly of the core prior to membrane budding, the size of RSV cores is positively correlated with particle size, consistent with the concurrent assembly and budding of RSV particles [12,15]. MMTV particles are also remarkable for the prominent glycoprotein knobs studding the lipid envelope. Strikingly, MMTV particles contain approximately 400 knobs, each of which has threefold symmetry, corresponding to roughly 1,200 copies of the Envelope (Env) glycoprotein [12].

Both MMTV and RSV particles contain protein cores that are irregular in shape and centrally placed within the lipid envelope, however it should be noted that up to 47% MMTV of particles contain more than one core [12,15]. In immature virus particles, the predominant protein within the core is Gag. MMTV Gag is composed of seven domains, which from N-terminus to C-terminus are MA (matrix), pp21 (phosphoprotein 21), p3, p8, n, CA (capsid), and NC (nucleocapsid) (Fig. 1.1C). As with all retroviruses, RSV Gag shares the same basic structural organization and is also composed of seven domains: MA, p2, p10, CA, SP (spacer peptide), NC, and PR (protease) (Fig. 1.1D). During, or shortly after immature particles are released from the cell, Gag is cleaved into its component
domains by the retroviral protease, resulting in the formation of the mature particle required for infectivity (Fig. 1.1B).

1.3.2 Genome Organization

The RNA genomes of all retroviruses share the same basic organization: 5’ R-U5-\textit{gag-pol-env}-U3-R (Fig. 1.2). During reverse transcription (described further in section 1.4.2), U3 is transposed upstream of the 5’ R region, while U5 is transposed downstream of the 3’ R region, such that the structure of the integrated provirus is U3-R-U5-\textit{gag-pol-env}-U3-R-U5. Together, the U3-R-U5 regions are respectively referred to as the 5’ and 3’ long terminal repeats (LTRs) and the 5’ LTR contains the promoter(s) that drive the production of viral RNA. In the case of MMTV, the LTRs are particularly long (about 1.2 kb in MMTV vs. 330 bp in RSV) due to the superantigen (Sag) coding sequence in U3 [1,16,17].

Simple retroviruses, as typified by RSV, encode little more than the three genes (\textit{gag}, \textit{pol}, and \textit{env}) required for replication (Fig. 1.2). As with all retroviruses, the Gag and Pol gene products are translated using the full-length RNA as a template, while the Env gene product is translated from a spliced viral mRNA (Fig. 1.2B) [1]. It should be noted that in addition to \textit{gag}, \textit{pol}, and \textit{env}, RSV encodes an additional \textit{src} gene, which is dispensable for replication but is required for oncogenic transformation \textit{in vivo}.

In contrast, “complex” retroviruses encode regulatory proteins in addition to \textit{gag}, \textit{pol}, and \textit{env}, at least one of which must have a virus-specific \textit{trans}-activating function, such as the HIV Rev protein (reviewed in [18]). Within the
Figure 1.2 MMTV and RSV Proviruses and RNAs. (A) Maps of the MMTV provirus and viral RNAs. The LTRs (U3, R, U5) are shown as white boxes. The hormone responsive element (HRE) in U3 is shown as a pointed black box and an arrow indicates the transcription start site. The unspliced RNA is shown with Gag, Gag-Pro, and Gag-Pro-Pol reading frames indicated by white boxes. Frameshifts are shown as broken lines between reading frames. Filled arrowheads indicate splice donor (SD) sites, while splice acceptor (SA) sites are indicated by open arrowheads. Spliced regions of the RNAs encoding Env and Rem are indicated by dashed lines. For clarity, the spliced RNA encoding the sag accessory gene is not shown. (B) Maps of the RSV provirus and viral RNAs are annotated as in (A). For clarity, the RNA encoding the src accessory gene is not shown.
last ten years, two groups independently reported the presence of a Rev-like protein in MMTV called Rem (regulator of export of MMTV mRNAs), which reclassified MMTV as a complex retrovirus (Fig. 1.1A) [19,20]. Another notable difference between RSV and MMTV is that the MMTV protease (pro) reading frame is -1 relative to the gag reading frame, making pro a separate gene between gag and pol. By contrast, RSV protease is in the same reading frame as gag, and is therefore considered a domain of the Gag gene product. MMTV also encodes two additional genes: a dUTPase (dut), which has an unknown function in virus replication, and superantigen (sag), which is required for infection in vivo [21].

Retroviral genomic RNAs (gRNAs) are packaged as a non-covalent dimer of identical 35S gRNAs, referred to as the 70S RNA. Dimerization is initiated by stem-loop structures in the 5’ end of the gRNA called the dimerization initiation site (DIS), and maintained by the dimerization linkage structure (DLS) [22]. For RSV, sequences between nt 208 – 274 and 400 – 600 have been implicated in dimerization [23,24,25]. The DLS of MMTV is less well-defined, however a putative sequence has been defined within the MA domain of the gag coding region [26].

1.4 Retrovirus Life Cycle

This dissertation will describe the retroviral life cycle beginning with the attachment and entry of virus particles into an uninfected cell (Fig. 1.3).
**Figure 1.3 MMTV and RSV Replication Cycles.** The early events of retroviral replication common to MMTV and RSV include receptor binding and entry (1), followed by reverse transcription (2), and trafficking of the pre-integration complex (PIC) to the nucleus (3). Late events include transcription and splicing of viral RNAs (4), nuclear export of unspliced RNAs (5) and translation of viral proteins (6). Following this point, events specific to MMTV are shown above the dashed line, while events specific to RSV are shown below the dashed line. These events include the selection of genomes for encapsidation (7), assembly of immature virus particles (8), membrane trafficking of Gag (9), and immature particle release (10). Maturation of virus particles (11) is common to both MMTV and RSV.
Accordingly, viral transcripts and proteins will be described at appropriate points in the replication cycle.

### 1.4.1 Receptor Binding & Entry (Fig. 1.3[1])

The entry of a retrovirus particle into an uninfected cell is initiated by the recognition of a cell surface protein by the surface (SU) domain of the viral Env protein. Interestingly, different strains of RSV are able to utilize different cellular receptors, encoded by the genes \textit{tv-a}, \textit{tv-b}, and \textit{tv-c}. For example, the Prague strain uses the \textit{tv-a} or \textit{tv-b} receptors, while the Schmidt-Ruppin strain is restricted to the \textit{tv-b} receptor [1]. The \textit{tv-a} gene encodes a low-density lipoprotein receptor, while the \textit{tv-b} gene encodes a protein called CAR1, a transmembrane protein related to the tumor necrosis factor receptor family of receptors [27,28].

In contrast, the MMTV Env protein recognizes murine transferrin receptor 1 [29]. MMTV has been reported to infect human cells, although MMTV Env does not promote internalization of the virus after binding the human transferrin receptor, suggesting that MMTV must use another receptor to infect human cells [30,31,32]. Fusion of the MMTV envelope with the membrane of the host cell requires a pH < 5, indicating that MMTV particles are internalized through receptor-mediated endocytosis and that fusion occurs within endosomes [32].

### 1.4.2 Reverse Transcription (Fig. 1.3[2])

The reverse transcription (RT) reaction occurs shortly after the release of the virus core into the host cell, and is mediated by the viral Pol protein. The
reaction begins with the synthesis of minus-strand DNA, which is primed using a specific tRNA molecule. The tRNA binds to a region of the genomic RNA near the 5’ end called the primer binding site (PBS), and is highly specific to each retrovirus. For example, MMTV uses the Lys\textsubscript{3} tRNA while RSV uses the Trp tRNA to prime minus strand DNA synthesis [33,34,35,36]. The details of the RT reaction have been thoroughly reviewed elsewhere [1], but briefly the tRNA primer is used to synthesize a short stretch of minus strand DNA, called the minus strand strong-stop DNA (–sssDNA), and the RNA template is degraded by RNaseH activity of Pol. The R region of the –sssDNA is then transferred to bind the R region at the 3’ end of the genome where it serves as a primer to synthesize the remainder of the –DNA. RNaseH degradation of the template is concurrent with minus strand synthesis with the exception of a short stretch of RNA near the 3’ end of the genome called the polypyrimidine tract (PPT), which is relatively resistant to RNaseH degradation. The PPT serves as a primer for the synthesis of a short region of positive-stranded DNA called the plus-strand strong-stop DNA (+sssDNA), which stops after the incorporation of the PBS and a short sequence of the tRNA primer. The +sssDNA then binds to complimentary sequences at the 5’ end of the –DNA, where it serves as a primer for the remainder of +DNA synthesis, completing the RT reaction.

1.4.3 Nuclear Trafficking of the PIC & Integration (Fig. 1.3[3])

After gaining entry to an uninfected cell, retroviruses must gain access to the nucleus to complete their replication cycle. The ribonucleoprotein complex
(RNP) containing viral proteins, nucleic acid (genomic RNA or double-stranded DNA), and cellular factors that traffics through the host cell prior to integration is called the pre-integration complex (PIC) [1]. Although the exact composition of the PIC may vary between retroviruses, MA, CA, and NC have been detected in the PIC and may play important roles in nuclear trafficking of the complex [1,37,38,39].

Once the PIC reaches the nuclear membrane, the DNA and integrase must gain access to the nucleus for integration to occur. Some retroviruses, such as HIV-1 and MMTV, can infect non-dividing cells, suggesting that the RNP required for integration is transported across the nuclear pore in an energy-dependent manner [40,41]. Interestingly, RSV can also infect non-dividing cells, although with lower efficiency than HIV-1 [42,43]. However, RSV integration occurs during S-phase and cell division may be required for efficient transcription of RSV RNAs, indicating that RSV may depend on cell division to carry out later parts of its life cycle [44,45].

Integration of the double-stranded DNA molecule contained within the PIC is accomplished by viral integrase (IN) through a series of two reactions. During the first reaction, two or three nucleotides are removed from the 3’ ends of the double stranded DNA to form invariant CA_{OH} 3’ dinucleotides [46,47]. This reaction occurs soon after the completion of the RT reaction, and marks the transition of the reverse transcription complex to the PIC [48]. Once the PIC has reached the interior of the nucleus, IN catalyzes the insertion of viral double-stranded DNA into the cellular chromosome. In this reaction, the processed 3’ OH
groups are coordinated in a staggered nucleophilic attack on the chromosomal DNA to cut the cellular DNA and simultaneously join the viral DNA to the newly cut 5' ends of the cellular DNA [48]. Cellular enzymes then repair the remaining nicks on the opposite strands.

Interestingly, retroviruses appear to have varying preferences for the site of integration within the host genome. For example, integration of HIV-1 is targeted to sites of active transcription via an interaction between IN and LEDGF/p75 (lens epithelium-derived growth factor), a cellular chromatin-binding protein [49,50,51,52]. However, it should be noted that determinants within Gag might also play a role in integration site selection [53]. Interestingly, RSV also displays a preference for integrating near transcriptionally-active genes, however the mechanism underlying this targeting is not known [49,52,54]. By contrast, the integration site selection exhibited by MMTV is, as far as can be assessed, completely without preference for transcriptional units or any of several other genomic features [52]. As a result, the integration of an MMTV provirus near a cellular oncogene is not a targeted event, but rather a random one resulting from high levels of MMTV replication and the absence of a barrier to superinfection, resulting in multiple integrations within a single cell [52,55].

1.4.4 Transcription & Splicing of MMTV and RSV RNA (Fig. 1.3[4])

Retroviruses do not encode a DNA-dependent RNA polymerase, and therefore transcription of viral RNA is mediated by cellular RNA polymerase II [56,57]. However, the promoter complex driving MMTV RNA production is multi-
faceted because while MMTV can undergo constitutive, low-level transcription in multiple cell types, transcription of MMTV RNA at high levels is both tissue- and hormone-dependent [21]. The TATA box for the hormone-responsive MMTV promoter resides at nucleotides -27 to -31, just upstream of the hormone responsive element (HRE) [58]. The HRE itself stretches from -59 to -202, and is bound by glucocorticoid receptors following stimulation with progesterone or dexamethasone, which drives transcription initiation from the 5’ end of the R region (Fig. 1.2A) [58,59,60,61,62].

MMTV also has two additional TATA boxes in U3 at positions -469 to -464 (P3) and -671 through -676 (P2) [63,64,65]. Transcription from the P2 promoter is repressed by two downstream negative regulatory elements, which may mediate cell type-specific repression of transcription at this promoter [65,66]. However, an upstream enhancer region positively regulates transcription from P2 and P3 [66]. It is also worth noting that MMTV also possesses an additional promoter in the env gene, which drives T-cell specific production of a sag mRNA [67,68]. As a result, this LTR promoter complex gives MMTV the ability to transcribe viral RNA in multiple cell types and tissues throughout the virus infection cycle.

By contrast, RSV possesses a single, strong promoter in U3. This promoter lies approximately 30 nucleotides upstream of the transcription start site, which begins at the 5’ terminus of the R region [69]. Interestingly, this promoter is functional in a variety of cell types, consistent with a lack of tissue-specific expression by RSV [70].
During infection, MMTV transcribes at least five RNA transcripts: one doubly spliced transcript encoding the Rem protein; two singly-spliced transcripts encoding the superantigen (Sag) protein; one singly-spliced RNA encoding the envelope glycoprotein (Env); and one full-length RNA that can be used to transcribe Gag, Gag-Pro, and Gag-Pro-Pol, or can be packaged into new virus particles as a gRNA (Fig. 1.2A) [21]. Like cellular mRNAs, viral RNAs are modified by the addition of a 5' 7'-methylguanosine cap and a 3' poly(A) tail [71,72,73,74]. Furthermore, like many cellular transcripts, retroviral RNAs are also subjected to splicing, however retroviruses must also overcome cellular roadblocks to export unspliced RNA from the nucleus.

The major MMTV splice donor site (SD) is a canonical GT nucleotide sequence in the 5' UTR just upstream of the gag initiation codon (Fig. 1.2A) [26,60]. Although the sequence upstream of the SD differs slightly from the classical (C/A)AGGU(A/G)AGU consensus sequence, the MMTV SD sequence is similar to sequences detected in cellular mRNAs [75]. All MMTV spliced RNAs are generated using this donor site with the exception of the sag transcript driven by the env promoter [19,20,26,60,67]. The splice acceptor sequence (SA) for the env mRNA is highly similar to the (C/U)_{10}NCAGG consensus sequence observed for cellular mRNAs, and splicing occurs one nucleotide upstream of the env initiation codon [60,75]. Similarly, splicing of sag transcripts driven by the 5' LTR promoter occurs between the SD in the 5' UTR and a 3' splice acceptor 72 nucleotides upstream of the 3' UTR and sag initiation codon [17]. In contrast, sag transcripts initiating from the env promoter utilize a splice donor site in env but the
same splice acceptor as transcripts driven from the 5' LTR promoter [76].

Transcripts encoding rem, on the other hand, are completely spliced using both pairs of SD and SA sites described above (Fig. 1.2A) [20].

In contrast to MMTV, RSV produces only two spliced mRNAs using a single 5' SD site. The SD site is located 17 nucleotides downstream of the gag initiation codon and is similar to the classical SD consensus sequence [77,78]. An important implication of the location of the SD site is that all RSV RNA species contain the same 5' UTR and the first 6 codons of the gag coding sequence (Fig. 1.2B) [79,80]. The env splice acceptor sequence is located at nucleotide 5,078 and follows the consensus (C/U)_10NCAGG SA sequence [79]. However, this SA site is inefficiently utilized due to a non-consensus branchpoint sequence [81]. The src splice acceptor lies at nucleotide position 7054 and is also inefficiently utilized due to upstream splicing inhibitor sequence combined with a nonconsensus polypyrimidine tract at the SA site [82]. Together, the suboptimal splicing of RSV RNA leads to the production of 71.2% unspliced RNA, 16% src mRNA, and 12.8% env mRNA [82].

1.4.5 Nuclear Export of Unspliced Retroviral RNAs (Fig. 1.3[5])

Cellular machinery generally blocks the nuclear export of unspliced mRNAs, however all retroviruses must export unspliced RNA from the nucleus for efficient replication. As a result, retroviruses have evolved a number of strategies to circumvent the cellular roadblocks to unspliced RNA export. For MMTV, the viral mRNA encoding Rem is likely to be the first RNA released into the cytoplasm
because it is the only completely spliced MMTV RNA. The Rem protein is comprised of three domains: an N-terminal 98 amino acid signal peptide (SP), the first 162 amino acids of the Env surface domain (ΔSU), and the C-terminal 41 amino acids of the Env transmembrane domain (ΔTM). SP contains the major motifs important for Rem function, including two nuclear localization signals (NLSs), a nucleolar localization signal (NoLS), a nuclear export signal (NES) and an arginine rich motif (ARM) RNA-binding domain [20]. After Rem is translated on the endoplasmic reticulum (ER) the signal peptide is cleaved, probably by signal peptide peptidase, and SP is retrotranslocated from the ER to the nucleus where it accumulates in nucleoli [19,20,83,84,85].

Because SP contains all of the signals for nuclear trafficking and RNA binding, it is thought to be the functional molecule that mediates nuclear export of suboptimally- and un-spliced viral RNA. Indeed, SP is sufficient to mediate luciferase expression by Rem-dependent reporter constructs [85]. However, it should be noted that mutating two glycosylation sites in the ΔSU domain of Rem blocks Rem-dependent expression of luciferase reporters without affecting SP cleavage [83], indicating that full-length Rem may play an important role in nuclear RNA export or translation.

The Rem SP recognizes the Rem-responsive element (RemRE) on the viral RNA, a 496-nucleotide sequence that spans the boundary between env and the 3’ untranslated region (UTR) [19,86,87]. As a result, all MMTV RNAs contain the RemRE. Deletions within this region abolish Gag expression from unspliced viral RNAs and expression of reporter proteins from unspliced RemRE-containing
constructs, indicating that a functional RemRE/Rem system is required for nuclear export of unspliced MMTV RNA [86,88].

Interestingly, the HIV-1 Rev protein or HTLV-1 Rex protein can functionally substitute for Rem in reporter assays, indicating that Rem mediates RNA export through a mechanism similar to Rev and Rex [88]. However, it is not known whether Rem, Rev, or Rex affect MMTV gene expression or RNA encapsidation. Given the similarities between Rem and Rev, it is not surprising that Rem-mediated export of unspliced RNA is dependent on CRM1 (chromosome region maintenance-1) and can be inhibited by dominant-negative CRM1 mutants or by treatment with leptomycin B, which irreversibly inhibits CRM1 [19,20,88]. However, nuclear export of the singly spliced env mRNA is largely unaffected by CRM1 inhibition, suggesting that the env transcript may also be exported through another pathway [20,86]. Although this pathway has not been elucidated, one possibility is that spliced MMTV mRNAs may be exported through the Tap(Mex67)/Dbp5 pathway, which mediates nuclear export of spliced cellular mRNAs (reviewed in [89]).

In contrast to MMTV, RSV does not encode a functional analog of HIV-1 Rev and must therefore use other mechanisms to export unspliced RNA. One mechanism may involve two cis-acting ~135 nucleotide direct repeat (DR) elements flanking the src gene. These DR elements are able to functionally substitute for HIV-1 Rev in reporter assays, and deletion of both DRs reduces, but does not eliminate, unspliced RSV RNA in the cytoplasm [90,91]. As a result, these DRs may constitute a constitutive transport element (CTE) similar to the
CTE in Mason-Pfizer Monkey Virus (M-PMV) [92]. Although the precise mechanism of DR-mediated RNA export is not known, this pathway is likely to depend on Tap and Dbp5 [93]. Interestingly, RSV Gag may also play a role in nuclear export of unspliced viral RNA for packaging. Gag traffics through the nucleus, and Gag mutants deficient in nuclear trafficking package significantly less genomic RNA than wild-type [94,95,96]. However, RNA exported by Gag may not be available for translation because reporter protein assays for Gag-mediated RNA export did not reveal any effect of Gag on reporter protein expression [93]. Together, these data suggest a model where unspliced RSV RNA destined for translation is exported through the Tap/Dbp5 pathway while Gag mediates the export of unspliced genomic RNA through the CRM1 pathway.

1.4.6 Translation of Retroviral Proteins (Fig. 1.3[6])

Cellular ribosomes mediate the translation of viral mRNAs. As mentioned above, retroviral RNAs are 5’ 7-methylguanosine capped and 3’ poly-adenylated, however retroviral RNAs face several barriers to efficient cap-mediated translation. Primarily, retroviral 5’ UTRs are relatively long and rich in secondary structure, features which both inhibit efficient translation [97,98]. Furthermore, the 5’ UTR of retroviral RNAs frequently contains cis-acting sequences required for other aspects of the virus life cycle, including the tRNA primer binding site required for reverse transcription (discussed in section 1.4.2), the DIS and DLS, the SD site, and the Ψ packaging signal (discussed in section 1.3.7) (reviewed in [99]). Despite these obstacles, however, retroviruses must efficiently translate
protein and have therefore evolved mechanisms to efficiently translate their RNAs.

One mechanism that several retroviruses use to circumvent these barriers to cap-dependent translation is to avoid cap-dependent translation altogether through the use of an internal ribosome entry site (IRES). These sequences, originally identified in the Picornaviridae, recruit the 43S ribosomal subunit independently of the 5' cap and eIF3 to promote translation [100]. Functional IRES sequences have been identified in the 5' UTRs of several retroviruses, including RSV and MMTV, suggesting that retroviruses may utilize cap-independent mechanisms to translate their RNA [101,102]. In support of this idea many retroviral proteases, including that of MMTV, are able to cleave eIF4G in vitro and in vivo, which inhibits cap-dependent translation [103]. However, the presence and activity of IRES sequences in retroviral RNAs has remained controversial due to false-positive results due to cryptic promoters or splicing of bicistronic vectors used to study IRES activity [99].

As mentioned above, spliced mRNAs direct the synthesis of Env and, in the case of MMTV, Rem and SAg. In contrast, unspliced RNA directs the translation of the Gag, Gag-Pro, and Gag-Pro-Pol polyproteins. For RSV, Gag and Pro are in the same reading frame and therefore all Gag proteins are actually synthesized as Gag-Pro fusion proteins. However, the Pol reading frame is -1 relative to that of Gag, requiring a ribosomal frameshift to produce Gag-Pol [104]. This frameshift event is mediated by an AAAUUUA "slippery sequence" and a complex downstream stem-loop structure that forms an RNA pseudoknot
Together, these features combine to achieve a 20:1 ratio of Gag:Gag-Pol [104].

By contrast, the MMTV Pro gene is in a -1 frame relative to Gag, and the Pol frame is -1 relative to Pro (-2 relative to Gag). As a result, MMTV requires two ribosomal frameshifts to produce the Gag-Pro-Pol fusion protein [107,108]. Similar to the Gag-Pol frameshift in RSV, the MMTV Gag-Pro frameshift requires a heptanucleotide slippery sequence (AAAAAAC) and a downstream RNA pseudoknot [107,109,110]. The Gag-Pol frameshift site uses a different heptanucleotide sequence (GGAUUUA) but is also dependent on an RNA pseudoknot 3' to the slippery sequence, indicating that Gag-Pol frameshifting occurs through a similar mechanism [107,108,111]. As a result, the MMTV Gag, Gag-Pro, and Gag-Pro-Pol proteins are produced in a ratio of 30:10:1 [107,112].

1.4.7 Selection of Viral RNA for Packaging (Fig. 1.3[7])

During infection, retroviruses package their own genomic RNA far in excess of non-specific cellular RNAs [113]. This specificity is conferred by a cis-acting RNA sequence called Psi (Ψ) and the NC domain of Gag. All retroviral NC domains contain one or two conserved motifs, called “Cis-His boxes”, that follow the sequence C-X2-C-X4-H-X4-C [114]. These residues form a highly structured “zinc knuckle” that binds a single zinc atom and interacts with nucleotides within the Ψ sequence [115,116,117,118,119]. Interestingly, the NC domain is responsible for recognizing a particular Ψ sequence. For example, HIV-1-based chimeric proteins where the HIV-1 NC domain was replaced with that of MLV,
selectively encapsidated MLV RNA [120,121]. Similarly, replacing the RSV NC domain with that of MLV also causes the resultant Gag chimera to package MLV RNA [122]. Together, these data indicate that NC is the major protein determinant of viral RNA packaging. However, other domains of Gag such as MA may also make small contributions to the selection of viral RNA for encapsidation [123,124].

The MMTV \( \Psi \) sequence has been mapped to the 5’ end of the genome. Early experiments where the MMTV 5’ UTR was replaced with that of RSV abolished encapsidation of retroviral RNA [125], suggesting that \( \Psi \) was at least partly contained within the 5’ UTR. More recent experiments have revealed that \( \Psi \) also extends through at least 120 nucleotides of the \( gag \) coding region [126,127], however the 5’ end of \( \Psi \) remains poorly defined.

Mutational analysis in the context of the first 400 nucleotides of Gag showed that R, U5, and the first 128 nucleotides upstream of \( gag \) were not sufficient for infectivity, but the entire 160 nt region upstream of \( gag \) restored infectivity [127]. This indicates that the 3’ end of the 5’ UTR is required for encapsidation of the viral genome, however 5’ truncations of the 5’ UTR were not tested [127]. Therefore, the location of MMTV \( \Psi \) can only be resolved to the entire 5’ UTR plus 120 nucleotides of \( gag \). One interesting implication of this finding, however, is that the \( \Psi \) signal overlaps the MMTV SD site, so the only RNA containing an intact \( \Psi \) signal is the full-length genomic RNA [26,60,127]. By contrast, the RSV \( \Psi \) signal spans 216 nucleotides between the primer binding site
and the SD in Gag, which implies that all RSV RNAs contain a functional \( \Psi \) sequence [128].

The fact that \( \Psi \)-containing RNAs serve two roles in retrovirus replication, as mRNA and genome, raises an interesting question: how does the virus “decide” whether to use an RNA for translation or encapsidation as a genome? In general, three models have been proposed as an answer to this question (Fig. 1.4) [129]. The first model predicts the presence of two completely separate pools of RNA: one destined for translation, and the other destined for packaging (Fig. 1.4A). Alternatively, a single pool of RNA could serve as a source of mRNA for translation or gRNAs for encapsidation without the requirement that the RNA be used for translation before it is encapsidated (Fig. 1.4B). In contrast, the third model predicts that a single pool of RNA must serve as an mRNA to direct the translation of Gag, and that this Gag could then bind the RNA from which it was translated in \textit{cis} (Fig. 1.4C). Interestingly, studies across the \textit{Retroviridae} provide support for all three models among different viruses.

Evidence that retroviruses use two completely separate pools of RNA comes from experiments conducted on MLV. In these experiments, MLV-infected cells were treated with the RNA polymerase II inhibitor actinomycin D, and viral RNA content and protein synthesis were monitored by metabolic labeling [130,131,132]. Strikingly, MLV protein synthesis and virus production persisted for up to 8 hours after actinomycin D treatment, however the virus produced under these conditions was largely devoid of genomic RNA, suggesting that protein translation is driven by a stable pool of cytoplasmic RNA, but newly-synthesized
**Figure 1.4 Potential fates of retroviral RNAs.** (A) Unspliced viral RNA is sorted into two separate pools: one destined for translation of Gag, Gag-Pro, and Gag-Pro-Pol, and the other for encapsidation. (B) A single pool of viral RNA is the source of both mRNA for translation and genomes for encapsidation. RNAs used for translation can subsequently be packaged into virus particles, however translation is not required for RNA to be encapsidated. (C) Unspliced RNA is exported from the nucleus and must undergo at least one round of translation before being encapsidated into virus particles. Figure adapted from [129].
RNA is packaged into virus particles [130,131,132]. By contrast, when cells expressing HIV-1 were subjected to the same conditions, HIV-1 virion RNA levels dropped in proportion to the intracellular RNA, suggesting that MLV and HIV-1 used different mechanisms to select genomic RNAs for encapsidation [132].

Alternatively, studies examining the packaging of heterologous RNAs have indicated that HIV-2 may predominantly encapsidate its RNA in cis. In these experiments HIV-2 “helper viruses” that were co-transfected with ψ-containing replication-deficient vectors packaged only helper RNA and not vector RNA, indicating that HIV-2 Gag preferentially encapsidates the RNA from which it was translated [133,134]. This cis encapsidation strategy used by HIV-2 is thought to confer packaging specificity for unspliced RNA because like RSV, all HIV-2 RNAs contain ψ sequences [135]. However, more recent analysis of single virion RNA content has indicated that HIV-2 may also efficiently encapsidate heterologous RNAs in trans [136].

The finding that HIV-2 can encapsidate RNA in trans is not entirely surprising because virtually all retroviruses are capable of trans encapsidation, as evidenced by the production of retroviral vectors using multiple viruses including MMTV and RSV [113,125]. As a result, the second model of RNA encapsidation may be applicable to many retroviruses (Fig. 1.4B). However, this model does not address how ψ-containing RNAs are sorted for translation or encapsidation.

Retroviral ψ sequences are rich in secondary structure, which is important for recognition of ψ by NC but inhibits ribosome scanning and decreases translational efficiency [137,138,139]. This suggests that RNAs containing
structurally-intact $\Psi$ signals may not be optimal for translation [139]. However, during translation initiation RNA secondary structure is melted by RNA helicases, including eIF4A, while other proteins, such as eIF4B, bind single-stranded RNA to prevent re-annealing of secondary structural elements (reviewed in [140]). As a result, mRNAs undergoing active translation may not be optimal candidates for encapsidation. Together, this suggests that retroviruses may have evolved mechanisms to separate mRNA destined for translation from that destined for packaging.

Interestingly, NMR structures of the 5’ end of the HIV-1 genome reveal that the $\Psi$ region may adopt different conformations that favor translation or encapsidation. In the conformation thought to promote translation, the $gag$ initiation codon resides in stem-loop (SL) 4 of the HIV-1 $\Psi$ sequence, which is isolated from other secondary structural elements [137,141]. Furthermore, in this conformation the DIS on SL1 base pairs with a sequence in the U5 region [137]. Because HIV-1 preferentially packages RNA dimers, RNAs with this conformation may not be available for packaging [137]. In contrast, the RNA may also adopt a structure where the $gag$ initiation codon in SL4 base pairs with the 5’ UTR, which may inhibit translation and leaves the DIS on SL1 exposed, which may promote genome dimerization and encapsidation [137]. Together, these data suggest that changes in RNA secondary structure may function to determine the fate of the viral RNA.

Although short fragments of the HIV-1 RNA are able to adopt these different conformations in vitro, cellular factors may help regulate RNA secondary
structure in vivo. For example, RNA helicase A (RHA), a member of the DExD/H box family of RNA helicases, promotes translation of several retroviral Gag proteins by binding a region of the 5' UTR called the post-transcriptional control element (PCE) [142, 143, 144, 145]. Moreover, the ATPase domain of RHA is required to promote HIV-1 Gag translation, suggesting that the helicase function of RHA may remodel HIV-1 RNA to promote translation [145]. In contrast, knockdown of another DExD/H family member, DDX24, increases HIV-1 Gag translation but decreases RNA encapsidation, suggesting that DDX24 helps the viral RNA adopt a conformation that favors encapsidation [146]. Together, these findings indicate that cellular helicases play an important role in determining whether retroviral RNAs are used for translation or encapsidation, however the precise mechanism by which multiple helicases exert different effects to determine RNA fate remains unknown.

Interestingly, non-helicase RNA-binding proteins also play a role in retroviral genome packaging. For example, the SG-associated protein Staufen-1 promotes HIV-1 capsid assembly and RNA encapsidation [147, 148]. Furthermore, another SG-associated protein, YB1, represses translation of RSV RNA, however the effect of YB1 on RSV encapsidation has not been elucidated [149]. MMTV is capable of packaging its RNA in trans and, interestingly, Ψ-containing RNA may be required for efficient virus assembly, suggesting that Ψ may play an important structural role in the assembly of immature virus particles [125]. However, the host factors involved in MMTV RNA encapsidation remain to be defined.
Given the important role of cellular factors in retroviral RNA encapsidation, it is not surprising that viral accessory proteins may also play an important role in selecting RNAs for packaging. Recent evidence indicates that in addition to exporting unspliced RNA from the nucleus for translation of Gag, HIV-1 Rev may also play an important role in genomic RNA encapsidation. Specifically, Rev enhanced encapsidation of heterologous, Rev response element (RRE)-containing RNAs that were exported through a Rev-independent pathway, suggesting that Rev may have additional roles in determining the fate of unspliced RNAs [150,151]. MMTV Rem has similar functions to HIV-1 Rev, however it is not known whether Rem influences MMTV RNA fate beyond the nuclear export of unspliced RNA.

1.4.8 Assembly of Immature Virus Particles (Fig. 1.3[8])

1.4.8.1 Intracellular Trafficking of Gag and RNA

The initiating event in the assembly of retrovirus particles is binding of the viral RNA by a dimer of Gag [152,153,154]. However, the subcellular location of this initial interaction appears to vary among different retroviruses. For example, genetic evidence indicates that RSV Gag mutants that fail to traffic through the nucleus package reduced amounts of genomic RNA, suggesting that Gag first interacts with the viral RNA in the nucleus [95].

In contrast, the Gag protein and RNA of feline immunodeficiency virus (FIV) accumulate on the cytoplasmic face of the nuclear envelope, suggesting that the interaction between Gag and the RNA occurs shortly after the RNA is
exported from the nucleus [155]. Similarly, colocalization between HIV-1 Gag and its RNA has been observed at a perinuclear or centrosomal site, and HIV-1 RNA also accumulates on the nuclear envelope [155,156]. Interestingly, HIV-1 RNA dimerization is thought to occur in the cytoplasm [157], suggesting that after the RNA is exported from the nucleus it may rapidly dimerize and be bound by Gag in a perinuclear location. On the other hand, MLV Gag and its RNA colocalize predominantly on the cytoplasmic face of endosomes [158]. While it is not known whether this is the site of the initial interaction between Gag and the RNA, it does suggest a mechanism by which Gag:RNA complexes may be transported to the membrane. Interestingly, for all of the C-type viruses discussed above some mechanism appears to restrict the formation of large complexes of Gag on the RNA until the initial Gag:RNA complex reaches the membrane.

Unfortunately, little information exists on the trafficking pathways used by Gag and RNA of B/D-type retroviruses. Assembling M-PMV particles are frequently observed in a pericentriolar distribution [159], however the intracellular distribution of MPMV RNA is not known. Similarly, the early assembly steps of MMTV have not been probed.

1.4.8.2 Particle Assembly

Following its translation the Gag polyprotein orchestrates the assembly of retroviral particles. As mentioned previously, MMTV assembles immature “Type A” virus particles in the cytoplasm of infected cells [160]. These particles have a characteristic double-ring morphology, are about 80 nm in diameter, and
consist of a radial arrangement of Gag with MA on the outside of the particle and NC/Pro/Pol on the interior [161]. Assembly of immature particles is driven by Gag-Gag interactions, which are spread across multiple Gag domains including p3, p8, n, CA, and NC [162,163]. Following assembly, these particles frequently bud into intracellular membrane-lined compartments such as multivesicular bodies, or alternatively, may be transported to the plasma membrane for release into the extracellular environment [160,164,165,166].

Interestingly, evidence from multiple retroviruses indicates that cellular factors play an important role in retrovirus assembly, however the cellular factors implicated in MMTV and indeed the precise subcellular location of MMTV assembly remain unknown. MPMV, which follows a replication pathway phenotypically similar to that of MMTV, assembles its capsids in a perinuclear location near the centrosome [167]. Nascent Gag transcripts are targeted to this location by a signal in MA called the cytoplasmic targeting/retention signal (CTRS) [168]. Strikingly, a single R55W mutation within the CTRS changes MPMV assembly from a B/D-type morphology to C-type morphology, although particles made with R55W Gag are non-infectious [168,169]. MMTV contains a putative CTRS within MA that is able to functionally replace the MPMV CTRS [168], however there are no reports of MMTV assembly occurring in a pericentriolar location. Rather, electron microscopy studies have observed clusters of incomplete MMTV capsids within cytoplasmic patches of granular, electron dense material [160]. At the time, it was proposed that the granular material was viral in
origin [160], however there is no evidence to indicate that this material is not of cellular origin.

One potential clue regarding the subcellular location of MMTV capsid assembly comes from the yeast Ty3 retrotransposon. Although Ty3 lacks an envelope protein, and therefore does not bud, it does assemble virus-like particles (VLPs) in the cytoplasm, similarly to MMTV [170]. Ty3 VLP assembly occurs in conjunction with cytoplasmic ribonucleoprotein complexes called retrosomes, which also contain cellular mRNA-binding proteins associated with stress granules (SGs) and processing bodies (P-bodies) (described further in section 1.5.2) [170,171]. Many metazoan viruses, including retroviruses, also interact with components of SGs and/or P-bodies to facilitate their replication (reviewed in [172]) and remarkably, electron micrographs of SGs reveal a striking resemblance to the granular material surrounding assembling MMTV capsids [160,173]. Together, these data suggest that MMTV assembly may occur near or within SGs or P-bodies.

In contrast to MMTV, RSV follows the C-type morphogenic pathway and assembles immature capsids at the plasma membrane in a process that is concurrent with budding. Interactions between Gag molecules are mediated by the multimerization interface (MI), which spans the p10 and CA domains [174,175], and the interaction (I) domain within NC [176,177]. However, despite the accumulation of RSV Gag at the plasma membrane, this is unlikely to be the location of the initial Gag-Gag interaction that starts the assembly process. In fact, wild-type RSV Gag can rescue the incorporation of a nuclear-restricted Gag
into virus particles and fluorescence complementation experiments reveal that Gag-Gag interactions occur in the nucleus [178]. Additionally, large complexes of Gag have been detected in the cytoplasm [179], suggesting that capsid assembly may actually be well underway before the nascent particle reaches the membrane. However, the factors that enable RSV to bud specifically from the plasma membrane remain to be elucidated.

In addition to Gag, RNA also plays an important structural role in the assembly of retrovirus particles. Indeed, HIV-1 Gag mutants incapable of binding RNA fail to produce stable virus particles [180], and RNase treatment disrupts mature murine leukemia virus (MLV) particles [181]. Furthermore RNA promotes the assembly of virus-like particles in vitro [182,183,184], however the requirement for RNA can be overcome by replacing the RNA-binding NC domain with a leucine zipper domain, which permits RNA-independent virus assembly [185,186], although mutants lacking an NC domain entirely fail to assemble [187,188]. As a result, these data indicate that RNA serves as a scaffold to organize Gag molecules into an assembling virus particle.

1.4.9 Membrane targeting of Gag (Fig. 1.3[9])

To spread infection to a new cell, retroviral Gag proteins and the viral RNA must eventually traffic to the plasma membrane. The MA domain of Gag mediates plasma membrane targeting, however the signals that allow MA to target Gag specifically to the plasma membrane are incompletely understood. Despite a lack of sequence conservation, the MA domains of diverse retroviruses
fold into a compact, globular structures consisting of four alpha-helices [189,190]. Moreover, basic residues within the MA domain are approximated during three-dimensional folding to produce a basic patch on the surface of the protein, which helps mediate membrane binding through electrostatic interactions [191]. Additionally, the MA domains of several retroviruses are modified by the addition of myristic acid, a C14 saturated fatty acid, that helps stabilize the interaction between MA and the plasma membrane [192].

The structure of MMTV MA has not been solved, however it is known that the MA domain is myristoylated [193,194]. Myristate is added co-translationally to the N-terminal glycine residue of MMTV Gag after the initiator methionine has been removed [194]. Although the cellular enzyme responsible for myristoylation of MMTV Gag has not been identified, the reaction is probably mediated by cellular myristoyl-CoA: protein N-Myristoyltransferase (reviewed by [195]). As with other retroviruses, myristoylation is important for the production of MMTV virions [196], however the precise mechanism underlying the role of myristate in MMTV replication has not been studied. For other retroviruses such as HIV-1, the myristic acid moiety is folded into a hydrophobic cavity within the MA globular domain, and is then exposed following Gag trafficking to the plasma membrane (reviewed in [192]). As a result, myristoylation plays an important role in HIV-1 assembly [197].

However, myristoylation may play a different role in the replication of B- and D-type retroviruses, such as MMTV, which assemble in the cytoplasm. Indeed, MPMV is myristoylated, and although this modification plays no role in
MPMV assembly, it is important for the endosome-mediated transport of immature MPMV particles to the plasma membrane for budding [198]. Interestingly, MMTV frequently buds into intracellular vesicles such as multivesicular bodies, suggesting that MMTV MA may preferentially target immature capsids to intracellular membranes, which are then transported to the plasma membrane [160,166,199].

In contrast to MMTV, RSV MA is acetylated, not myristoylated, and therefore lacks any fatty acid modifications that contribute to membrane binding [193,200]. Rather, the membrane-binding domain of RSV Gag is spread across the first 87 amino acids of the MA domain [201,202]. Membrane binding is dependent on 11 basic residues scattered throughout MA, four of which (K23, K24, K67, and K72) fold to form a basic patch on the surface of MA [191]. However, the basic residues in MA do provide some functional redundancy, as a loss of only one residue generally results in only a partial budding defect [203]. Conversely, mutating two acidic residues to basic residues (E25Q/E70Q) significantly enhances the rate of RSV budding, suggesting that increasing the positive charge of the MA domain causes the protein to be more strongly localized to the plasma membrane [203]. Together, these results suggest that the interaction between RSV MA and the plasma membrane may be driven predominantly by electrostatic interactions.

However, what is less clear is how RSV MA is targeted specifically to the plasma membrane and not intracellular membranes. Interestingly, deleting the fourth alpha helix of RSV MA causes Gag to accumulate on intracellular
membranes, indicating that the fourth helix of MA helps confer specificity for the plasma membrane [204]. The N-terminal lysine residue (K72) of helix 4 forms part of the basic patch on MA, suggesting that deletion of helix 4 may disrupt the structure of the basic patch, thereby preventing RSV MA from recognizing a cellular factor specific to the plasma membrane [189]. K72Q MA mutants exhibit a partial loss of virus production, however the effect of mutating all four basic patch residues was not examined [189,203].

In other retroviruses the basic patch on MA serves as an important determinant of plasma membrane-specific MA localization. For example, in HIV-1 a basic cleft on the surface of MA binds the acidic phospholipid phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂], which is concentrated on the plasma membrane. Depletion of PI(4,5)P₂ leads to an accumulation of Gag on intracellular membranes, similar to the phenotype observed with deletion of helix 4 from RSV MA [204,205,206]. Although the precise function of the basic patch in RSV MA is not known, these data suggest that the basic patch on RSV MA may interact with PI(4,5)P₂ or another plasma membrane-specific acidic phospholipid to mediate specific targeting of Gag to the plasma membrane.

1.4.10 Virus Particle Release & Maturation (Fig. 1.3[10], [11])

The release of retrovirus particles requires several steps including the budding of the immature particle into the host cell plasma membrane, incorporation of the Env glycoprotein into the particle, and ultimately scission of the bud from the membrane. Together, these steps result in the release of the
enveloped particle into the environment. Evidence from HIV-1 suggests that CA-CA interactions drive the induction of cell membrane curvature by forcing MA hexamers into a curved lattice, which in turn changes the conformation of the membrane [207,208,209,210]. However, it should be noted that bud formation is a complex process that also involves rearrangement of the cellular actin cytoskeleton and recruitment of membrane microdomains to help facilitate the formation of curved membranes [209,211]. In addition to mediating topological changes of the cellular membrane, the MA domain also helps efficiently incorporate Env glycoproteins by interacting with the cytoplasmic C-terminal tail of the Env transmembrane domain [212,213]. However, RSV is still capable of producing infectious virus particles even when the C-terminal tail of Env has been deleted, suggesting that retroviruses may differ in the efficiency of Env incorporation required for infectivity [214].

The final steps of virus budding and scission are mediated by the cellular ESCRT (endosomal sorting complex required for transport) complex, which is recruited to sites of virus budding by the Gag late (L) domain. Late domains are short sequences which follow one of several amino acid sequence, including P(T/S)AP, YP(Xₙ)L, and PPₓY [215]. The RSV L domain consists of a PPPY sequence in p2 [216,217]. In contrast, the L domain of MMTV has yet to be defined, however a PSAP sequence in CA may serve as a late domain.

Following the release of the immature virus particle, Gag must be cleaved into its component domains by the viral protease to produce an infectious particle. For RSV, this process is concurrent with, or occurs just after budding [218].
contrast, MMTV undergoes significant proteolytic processing intracellularly, with only the final cleavage between CA and NC occurring after the release of the virus particle from the cell [112,196,219]. Proteolytic processing of Gag results causes a major change in the arrangement of proteins within the particle, which culminates with the formation of a mature, infectious virion (Fig. 1.3).

1.5 Cellular Factors & Retroviral Replication

1.5.1 The Nucleolus

The nucleolus is one of the most easily visible subcellular structures and indeed, nucleoli were first described by Rudolph Wagner in 1835, and subsequently by Gabriel Valentin, who named the structure in 1836 [220]. Accordingly, with over 150 years of history nucleoli are one of the best-studied subnuclear compartments. The classical, and best-described, function of the nucleolus is the synthesis of the 28S, 18S and 5.8S ribosomal RNAs (rRNAs), and the assembly of these RNAs and free ribosomal proteins into ribosomal subunits. However, in the past few decades it has become clear that the nucleolus also serves a variety of other functions including cell cycle regulation, nuclear export of mRNAs, and cellular stress responses [221,222,223,224,225]. As a result, it is not surprising that mass spectrometry analysis of purified nucleoli has identified over 4,700 proteins, only a minority of which have known functions in rRNA synthesis [226].

Ultrastructurally, nucleoli are divided into three distinct regions based on their appearance by electron microscopy: the fibrillar center (FC), the dense
fibrillar component (DFC), and the granular component (GC) (reviewed in [227]). Strikingly, these morphological zones also carry functional implications. Chromatin-containing the genes for rRNA are located in the FC, rRNA synthesis occurs either within the FC or on the FC/DFC border, modification and processing of rRNAs occurs within the DFC, and assembly of ribosomal subunits occurs within the GC [228].

Because rRNAs are essential for ribosome biosynthesis, and therefore cell growth, nucleoli also play an important role in regulating cell growth by regulating rRNA synthesis. For example, a nucleolar protein called PHF6, which is frequently mutated in adult T-cell lymphoblastic leukemia, was recently found to inhibit rRNA synthesis by binding to upstream binding factor (UBF) on the rDNA promoter [225]. Furthermore, nucleoli are also intimately involved in the regulation of cell cycle arrest and apoptosis through the p53 pathway. Many ribosomal proteins have “extraribosomal” functions, and frequently localize to the nucleolus when not incorporated into a ribosome. However, during stress several ribosomal proteins including RPL5 (ribosomal protein, large 5), RPL23, and RPL11 translocate from the nucleolus to the cytoplasm where they interact with MDM2 (murine double minute 2) to prevent MDM2-mediated ubiquitination of p53 [229,230,231,232]. This ubiquitination typically results in p53 degradation, so by interacting with MDM2 RPL5, RPL23, and RPL11 stabilize p53 levels, resulting in cell cycle arrest and/or apoptosis [233]. However, the nucleolar PICT1 protein binds and retains RPL11 in the nucleolus, thereby preventing RPL11 from
interacting with MDM2 [223,224]. As a result, one major function of nucleoli may be to regulate cell growth and division during cellular stress.

Given the pleotropic role of nucleoli in cellular physiology, it is perhaps not surprising that many viruses interact with nucleoli to facilitate virus replication or manipulate the cellular environment (reviewed in [234,235]). Most, if not all, viral and cellular proteins are targeted to the nucleolus by a NoLS. While these signals vary in length, they are generally rich in lysine and arginine, however the exact mechanism by which these sequences target proteins to the nucleolus remains largely unknown [236]. The first retroviral gene product known to traffic through the nucleus is the HIV-1 Rev protein, which contains a RRNRRRRWRERQRQI NoLS [237,238,239,240]. Interestingly, HIV-1 RNA is cleaved by nucleolar-localized ribozymes, and electron microscopy studies have visualized HIV-1 RNA in nucleoli, suggesting that Rev may interact with the viral RNA in nucleoli [241,242]. Strikingly, Rev also relocalizes Crm1 and the nucleoporin Nup98 to nucleoli, suggesting that nucleoli may be a major site of Rev-mediated viral RNA export [243]. In addition to Rev, RNA export proteins from a number of other complex retroviruses, including HTLV-1 Rex, Jaagsiekte sheep retrovirus (JSRV) Rej, Bovine immunodeficiency virus (BIV) Rev, and MMTV Rem also localize to nucleoli, indicating that nucleolar trafficking may be an important step in the nuclear export of unspliced retroviral RNAs [84,244,245,246,247].

Interestingly, retroviral Gag proteins may also transiently traffic through the nucleus. In addition to RSV, recent work has detected Gag proteins from several retroviruses in the nucleus, including HIV-1, FIV, and MLV, and strikingly, FIV Gag
accumulates in subnuclear structures that morphologically resemble nucleoli [248,249,250,251]. Moreover, the C-terminal fragment of nucleolin, a resident nucleolar protein, inhibits the assembly of several retroviruses, suggesting a potential role for nucleolin in retrovirus assembly [252]. However, whether nucleolar trafficking of Gag proteins is common among retroviruses and indeed, whether this trafficking step is important for virus replication, remains to be determined.

1.5.2 mRNA Granules

1.5.2.1 Biology of SGs

As their name suggests, stress granules form in response to translational repression during periods of cellular stress including, but not limited to, heat shock, ultraviolet irradiation, oxidative stress, and viral infection [253]. The discovery of SGs is typically attributed to Lutz Nover who, in 1983, described the formation of cytoplasmic granules in Peruvian tomato cells exposed to heat shock [254]. Nover and colleagues (1989) subsequently reported that heat shock caused a reduction in polysome-associated mRNA, coincident with the accumulation of mRNA in heat shock granules [255]. However, these results were not entirely correct. A more recent report indicates that while plants do form SGs following heat shock, they are distinct from heat shock granules and, moreover, that heat shock granules do not contain poly-adenylated mRNA [256].

As a result, the true history of SGs as messenger RNA-containing RNPs begins in 1999, when Kedersha and colleagues reported that stress-induced
translational arrest coincided with the accumulation of poly-adenylated mRNA in cytoplasmic granules containing three mRNA-binding proteins: T-cell Intracellular Antigen cytotoxic granule-associated RNA-binding protein-1 (TIA1), TIA1 cytotoxic granule-associated RNA binding protein-like-1 (TIAR), and Poly(A)-binding protein (PABP) [257]. Importantly, this work was also the first to link translational arrest induced by the phosphorylation of eukaryotic initiation factor 2α (eIF2α) on serine 51 to the formation of SGs [257,258], which provided important insight into the role of SGs as sorting centers for translationally-repressed mRNAs.

Following stress, the formation of stress granules most commonly occurs through a mechanism dependent on the phosphorylation of eIF2α, although exceptions do exist [259,260]. The phosphorylation of eIF2α on S51 is accomplished by one of four kinases: protein kinase R (PKR), which senses double-stranded RNA [261]; heme-regulated inhibitor (HRI), which responds to oxidative stress, heat shock, and osmotic stress [262,263]; pancreatic endoplasmic reticulum eIF2α kinase (PERK), a sensor of the unfolded protein response [264]; and general control nonderepressible-2 (GCN-2), which recognizes an accumulation of uncharged tRNA [265]. During translation initiation, the 40S ribosomal subunit bound to an mRNA is charged with the initiator Met-tRNAi by eIF2, which consists of α and β subunits bound to a central γ subunit. The Met-tRNAi is bound by the γ subunit of eIF2, which also binds GDP or GTP, although eIF2-GTP has a far higher affinity for the Met-tRNAi. Accordingly, the formation active eIF2-GTP-Met-tRNAi complex, called the ternary complex, is dependent on the exchange of GDP for GTP on eIF2γ by eIF2B [140].
Phosphorylation of eIF2α on S51 blocks the interaction with eIF2B, leading to a decrease in ternary complex formation and a general inhibition of translation [266], which, in turn, promotes the formation of SGs. As a result, mRNAs contained within SGs are bound by stalled 48S preinitiation complexes, but not the 60S ribosomal subunit [259]. Interestingly, however, not all mRNAs are recruited to SGs equally. For example, mRNAs encoding heat shock proteins are selectively excluded from SGs [267,268], however the mechanisms governing the recruitment of mRNAs to SGs are not well understood.

In addition to mRNAs, many proteins also localize to SGs (almost 80 as of 2009) (Table 1) [269]. Interestingly, the protein composition of SGs can vary significantly depending on the type of stress, however several proteins including components of the 48S preinitiation complex, TIA1, and PABP-1 are invariantly present within SGs [270]. Moreover, the overexpression of many SG-associated proteins including TIA1, tristetraprolin (TTP), and Ras GTPase activating-protein (SH3 domain) binding protein-1 (G3BP1) induces the formation of SGs (Table 1.1) [271,272,273]. These proteins frequently contain both RNA-binding and homo-oligomerization domains and nucleate the formation of compositionally normal SGs and are dependent on eIF2α phosphorylation, probably by PKR [274]. As a result, inducing SG formation by overexpressing SG-associated proteins has been an important tool in studying SG biology.

It is important to note that SG-associated proteins play an important role in the structure and function of SGs. Following translational repression mRNA-binding proteins that also contain strong homo- and hetero-oligomerization
domains, including TIA1 and G3BP1, mediate the nucleation of microscopically visible SGs [275]. However, other RNA-binding proteins such as zipcode binding protein 1 (ZBP1) also localize to SGs without influencing SG nucleation. Instead, ZBP1 is localized to SGs through its interactions with zipcode-containing RNAs, and ZBP1 acts to stabilize these transcripts within SGs [268]. As a result, mRNA-binding proteins may localize to SGs during stress and interact directly or indirectly with SG-associated proteins without nucleating SG formation per se.

Interestingly, many proteins and mRNA transcripts contained within SGs are highly dynamic, and rapidly cycle between SGs and the cytoplasm. For example, fluorescence recovery after photobleaching (FRAP) experiments revealed complete recovery of TIA1, TTP, and G3BP1 within 30 seconds of photobleaching [274]. In contrast, PABP1 exhibits only ~60% recovery 30 seconds post-bleaching, suggesting that PABP1 may be part of a different mRNP complex within SGs than TIA1, TTP, or G3BP1 [274]. Furthermore, other proteins such as Fas-Activated Serine/Threonine phospho-protein (FAST) exhibit very slow dynamics, with negligible recover after 30 seconds [274]. An important implication of these experiments that different proteins play different roles within SGs. For example, TIA1 is thought to help recruit translationally-repressed mRNAs to SGs, which is consistent with the rapid shuttling of the protein between the cytoplasm and SGs. In contrast, the slow recovery of FAST fluorescence within SGs suggests that this protein may act as a scaffold to which other SG-associated proteins bind. The intermediate recovery time of PABP1 likely results from the tight binding of PABP1 to the poly(A)-tails of mRNAs (kD = 5 nM) [288],
Table 1.1 SG, P-body (PB), and RISC proteins used in the experiments described in this dissertation

<table>
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<th>Protein</th>
<th>Function</th>
<th>SG/PB/RISC Localization</th>
<th>SG/PB/RISC Nucleation</th>
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which may limit recovery of PABP1 fluorescence to the rate of mRNA movement into SGs. This interpretation is consistent with FRAP studies using MS2-GFP to label RNAs within SGs. Strikingly, these studies revealed the presence of two pools of RNA within SGs, with half-times of recovery of 2 and 58 seconds, respectively [289]. As a result, mRNAs may cycle rapidly through SGs, or may be stored in SGs for longer periods of time.

The rapid kinetics of RNA movement into and out of SGs raises important questions regarding the fate of the RNA after leaving the SG. One possibility is for the RNA to return to a translationally active state. Interestingly, treating stressed cells with translational inhibitors such as cyclohexamide, which blocks elongation by inhibiting ribosome translocation thereby trapping translationally-active mRNAs on polysomes [290], causes SGs to disassemble despite continued stress [278,289]. As a result, SGs are highly dependent on a pool of mRNAs exiting translation. Furthermore, this result suggests that RNAs contained within SGs may rapidly return to active translation on polysomes [289]. An alternate possibility, however, is that trapping mRNAs on polysomes simply stops the flow of mRNA into SGs, and that the mRNAs within SGs are degraded. However, imaging experiments revealed the persistence of SGs despite actinomycin D treatment, suggesting that the majority of mRNAs within SGs are not targeted for degradation [289]. Despite this important insight, the mechanisms that regulate which mRNAs exit SGs and return to translation remain poorly understood.

One hint towards understanding how mRNAs are sorted within SGs comes from the observation that SGs themselves are highly dynamic and rapidly traffic
through the cell along microtubules [291]. Furthermore, SGs often split into smaller granules, fuse into larger granules, or disperse entirely [274,291]. These splitting, fusion, and dispersion events are thought to represent sorting of the mRNA contents of SGs [274]. Moreover, SGs frequently “dock” or fuse with P-bodies, which contain many mRNA degradation enzymes (discussed in detail in section 1.5.2.2) [274]. These interactions are thought to be required for the transfer of mRNA between SGs and PBs, and indeed overexpression of TTP, which is associated with both SGs and PBs and promotes RNA degradation, increases docking and fusion between SGs and PBs [274].

Together, these data support a model where SGs function as dynamic sorting centers for mRNA during stress. This model predicts that following the induction of stress, such as heat shock, “housekeeping” mRNAs not needed to survive the stress are translationally-repressed and targeted to SGs, which allows the preferential translation of mRNAs needed for cell survival, such as transcripts encoding heat shock proteins. Within SGs, housekeeping mRNAs undergo a dynamic sorting process which can result in transcripts being returned to translation on polysomes, stored in a translationally-repressed state in SGs or elsewhere, or passed to P-bodies for degradation. As a result, SGs represent an important mechanism by which cells can rapidly alter their protein expression profile in response to changing environmental conditions.
1.5.2.2 P-bodies and RISCs

The history of P-bodies is somewhat less convoluted than that of SGs. In fact, cytoplasmic granules, now known to be P-bodies, containing the murine 5’ → 3’ exonuclease Xrn1p were first described in 1997 [292]. However, the functional implications of these complexes were not appreciated until December of 2002 when three groups independently reported that the human mRNA decapping enzymes Dcp1 and Dcp2 also localized to small foci containing Xrn1p and the LSm1-7 complex, which is involved in mRNA degradation [293,294,295]. At the same time, Eystathioy et al. reported that antibodies from patients with primary biliary cirrhosis reacted with GW182, a 182 kDa protein with multiple glycine-tryptophan (GW) repeats, that localizes to small cytoplasmic granules [296]. Furthermore, microarray analysis revealed that GW182 complexed with mRNA, indicating that the observed GW182 foci were actually RNPs [296]. Subsequent experiments demonstrated that GW182 foci also contained Dcp1 and LSm4 [282], indicating that the GW bodies described by Eystathioy et al. were the same as the previously described Dcp1/2 / Xrn1 / Lsm1-7 complex. Because virtually all of the proteins found in these complexes were associated with mRNA degradation (Table 1.1), these complexes were assumed to be the site of mRNA degradation.

Like SGs, P-bodies contain translationally-repressed mRNA, however unlike stress granules, P-bodies are present under steady-state conditions although P-bodies do increase in size and number following cellular stress [274]. The observation that P-bodies are also larger and more abundant in yeast strains lacking key components of the mRNA decapping complex suggests that one
function of P-bodies is to degrade translationally-repressed mRNA [297].

However, not all transcripts within PBs are targeted for degradation and, in fact, mRNAs may exit P-bodies and return to translation on polysomes although these mRNAs may only represent about 20% of RNAs contained within P-bodies [298,299].

Interestingly, the cis- and trans-acting elements involved in targeting mRNAs to P-bodies are somewhat better understood than is the case with SGs. For example, mRNAs containing destabilizing AU-rich elements (AREs) in their 3’ untranslated regions (UTRs) can be targeted to P-bodies by TTP and its closely related family member BRF-1 [300]. Interestingly, either the N-terminal or C-terminal domain of either protein, when tethered to a transcript, is sufficient to target the transcript to P-bodies [300]. Moreover, TTP can actually nucleate the formation of a P-body on a transcript to which it is bound [300], suggesting that TTP and BRF-1 may serve a scaffolding function to recruit other P-body proteins to a transcript destined for degradation or long-term storage. Furthermore, at least in yeast, the nonsense-mediated decay proteins Upuf1p, Upuf2p, and Upuf3p all localize to P-bodies and transcripts bearing premature stop codons accumulate in P-bodies [301], suggesting that P-bodies play an integral role in the nonsense-mediated decay pathway. More recent data in human cells suggests that microscopically detectable P-bodies may not be required for nonsense-mediated decay [302], although these results do not exclude the possibility that nonsense-mediated decay may still occur in P-bodies.
Transcripts targeted for micro-RNA (miRNA)-mediated transcriptional silencing may also be targeted to P-bodies [303]. P-bodies contain RNA-interference (RNAi) effector molecules including Argonaute2 (Ago2) [304], a key component of the RNA-induced silencing complex. Interestingly, Ago2 mutants deficient in the ability to bind miRNAs are unable to localize to P-bodies, suggesting that miRNA binding is required for localization of Ago2 to P-bodies, and that P-bodies may be the location of Ago2-mediated translational repression [304]. However, the precise role of P-bodies in miRNA-mediated silencing is unclear. In *Drosophila*, GW182 and the DCP1:2 complex are required for efficient miRNA-mediated silencing [305]. Because knockdown of key P-body components including GW182, DCP1, or DCP2 inhibits miRNA-mediated gene silencing [305], these results suggest that P-bodies *per se* may be required for RNAi. However, other data suggest that RNAi occurs in RNPs distinct from P-bodies. Specifically, Gibbings *et al.* recently reported that GW182 and Ago2 form complexes on endosomal membranes that are distinct from P-bodies and that purified exosomes, which are derived from multivesicular bodies, are enriched in miRNAs and RISC components but not P-body proteins [306]. Furthermore, disrupting the endosomal sorting pathway inhibited miRNA-mediated silencing, which indicates that RNAi may occur on intracellular membranes such as multivesicular bodies [306].
1.5.2.3 Viral interactions with SGs, PBs, and RISCs

In addition to playing a crucial role in regulating cellular mRNAs, SGs and P-bodies may also represent an important cellular defense against infection by RNA viruses. Accordingly, many proteins with antiviral properties, including G3BP1, Y-box binding protein-1 (YB1), and apolipoprotein mRNA editing enzyme catalytic peptide-like (APOBEC) family members, localize to SGs and P-bodies (Table 1.1) [279, 287, 307]. However, many viruses have evolved mechanisms to circumvent, disable, or even hijack this cellular defense pathway to facilitate their own replication.

One mechanism by which RNA viruses hijack the cellular stress response is to induce the formation of SGs to inhibit cellular translation and promote the translation of viral proteins. Several viruses from diverse families utilize this approach, including Semliki forest virus (Alphaviridae) [308], mammalian orthoreovirus (Reoviridae) [309], and mouse hepatitis corona virus (Coronaviridae) [310]. Additionally, respiratory syncytial virus induces the formation of SGs during virus infection, however the virus appears more dependent on G3BP1 than SGs per se as knocking down G3BP1 decreases virus replication, but inhibiting SG formation by knocking down PKR does not affect replication [311, 312]. These data suggest that respiratory syncytial virus has evolved mechanisms to circumvent SG-mediated restriction of virus replication.

On the other hand, many RNA viruses inhibit the formation of SGs to disable the SG-mediated cellular defense pathway. For example, Poliovirus (Picornaviridae) inhibits formation of SGs by 3C protease-mediated cleavage of
G3BP1, which is required for efficient virus replication [279]. Similarly, West Nile virus and dengue virus (Flaviviridae) inhibit SG formation, ostensibly by interacting with TIA1 [313]. Mammalian retroviruses also inhibit the formation of SGs as the Tax protein of Human T-cell Leukemia Virus type-1 (HTLV-1) interacts with histone deacetylase 6 to inhibit the formation of SGs [314]. Similarly, HIV-1 infection inhibits SG formation and although the mechanism is unknown, one report suggests that this inhibition may be mediated by the Gag protein [172]. Interestingly, the HIV-1 protease also cleaves eIF4G and PABP [103,315], both of which are required for efficient translation. Knockdown of several translation initiation factors inhibits the formation of SGs [316], suggesting that HIV-1 protease may inhibit the formation of SG formation by cleaving eIF4G or PABP.

Despite the general inhibition of SG formation by HIV-1, the virus does depend on the SG-associated protein Staufen-1 for efficient replication. HIV-1 Gag, viral RNA, and Staufen-1 colocalize in Staufen-1 HIV-1-dependent RNP (SHRNPs), which are distinct from SGs and P-bodies, and modulation of Staufen-1 expression alters HIV-1 genomic RNA encapsidation and virion assembly [147,148,317,318]. However, it is not known whether other SG-associated proteins also present in these SHRNPs and, if so, what role these proteins play in HIV-1 replication.

Surprisingly, HIV-1 Gag also interacts with the P-body proteins typically associated with RNAi-mediated silencing to promote virus production. For example, HIV-1 Gag recruits Ago2 to unspliced viral RNAs through a mechanism that is independent of both miRNA binding and P-bodies [319]. Strikingly, the
Gag-mediated recruitment of Ago2 to Ψ-containing RNAs did not interfere with RNA translation, however depletion of Ago2 significantly reduced virus particle production [319]. Although the mechanism by which Ago2 affects particle production is not known, this interaction is conserved with primate foamy virus-1 (PFV-1) suggesting that diverse retroviruses have evolved to use Ago2 to facilitate virus replication [319]. Furthermore, HIV-1 also interacts with Ddx6, a DEAD-box RNA helicase implicated in RNAi, in an RNA-dependent manner [320]. As with Ago2, this interaction is required for efficient virus production, however unlike Ago2, Ddx6 is not encapsidated into virus particles, indicating that the Gag-Ddx6 complex is an assembly intermediate [319,320]. Together, these data indicate that metazoan retroviruses interact with several P-body- and SG-associated proteins to facilitate virus assembly, however it remains to be determined whether all of these proteins are present in a single complex, or whether assembly progresses through a series of intermediate complexes with varying protein compositions.

Data from the retrovirus-like Ty1 and Ty3 yeast retrotransposons indicate that retrotransposon assembly occurs in distinct cytoplasmic granules called “retrosomes”, which contain multiple SG- and P-body-associated proteins required for efficient retrotransposition [170,171,321,322]. Furthermore, the mammalian LINE-1 retrotransposon localizes its RNA and proteins to large cytoplasmic complexes containing SG and P-body proteins, which are thought to represent LINE-1 replication intermediates [323]. As a result, these data suggest that reverse transcribing elements (i.e. retroelements), including retroviruses and
retrotransposons, may commonly hijack SG and/or P-body proteins to facilitate their own replication. However, the role of many SG- or P-body-associated proteins in promoting retrovirus and retrotransposon replication has not been intensely investigated.

Conversely, SG and P-body proteins may also play a conserved role in restricting retroelement replication. The best-studied group of proteins with such a role is the APOBEC family of mRNA editing enzymes. These enzymes are encapsidated into retrovirus particles and inhibit replication by causing C \( \rightarrow \) U transition mutations (reviewed in [324]). However, recent evidence indicates that APOBEC-mediated inhibition of HIV1 (in the absence of Vif) is independent of P-bodies [325]. Interestingly, increased expression of Mov10, a putative RNA helicase that localizes to P-bodies, restricts the replication of HIV-1 and several other retroviruses [326,327,328], however some data show that Mov10 is also required for efficient HIV-1 replication [329]. Furthermore, recent data indicate that Mov10 also restricts the replication of endogenous retroelements [326,330], suggesting that Mov10 has an evolutionarily conserved role in restricting the replication of retroviruses and retrotransposons.

The cytoplasmic assembly pathway followed by MMTV is morphologically similar to that of endogenous retrotransposons in yeast (i.e. Ty3) and mammals (i.e. LINE-1), and shares many key steps with C-type retroviruses, including the sorting of unspliced viral RNA for translation or encapsidation (Fig. 1.5). As a result, MMTV may interact with many of the same SG- and P-body-associated proteins as other retroviruses and retrotransposons, however the role of these
proteins in MMTV assembly has not been determined. However, due to the spatial and temporal separation of MMTV assembly and budding, MMTV represents an ideal model system to study the role of these RNA-binding proteins in retrovirus assembly.

1.6 Overview

The work presented in this dissertation spans two evolutionarily diverse retroviruses, MMTV and RSV, and discusses the role of several host factors and/or subcellular compartments on retrovirus replication. As a result, data chapters are organized by subcellular location, beginning with the innermost part of the cell (i.e. the nucleus and nucleolus), moving outwards through the cytoplasm, and ending at the plasma membrane. However, the order of these chapters should not be taken to imply the order of Gag trafficking during virus assembly. Moreover, while evidence in the literature suggests that diverse retroviruses interact with conserved sets of cellular factors, the data presented in this dissertation should not be interpreted as a conserved set of interactions applicable to all retroviruses. As a result, factors implicated in the assembly of MMTV may, or may not, be implicated in RSV assembly as well, and vice versa. Unfortunately, these studies of comparative retrovirology fall beyond the scope of this work and must therefore be left for future researchers. Rather, each virus was chosen, based on its specific attributes and characteristics, to answer specific questions about retroviral biology.
Figure 1.5. Common features of C-type retrovirus, B/D-type retrovirus, and retrotransposon assembly pathways. C-type and B/D-type retroviruses share important features of their assembly pathways, including the sorting of unspliced viral RNA for use as an mRNA or encapsidation into virus particles as a genome. Similarly to B/D-type retroviruses, retrotransposons assemble in cytoplasmic RNPs but unlike retroviruses, retrotransposons do not bud from the cell and instead return to the nucleus where they are re-integrated into the chromosome. Accordingly, studies on B/D-type retroviruses may also provide insight into the replication of C-type retroviruses, as well as retrotransposons.
Chapter 2

Nucleolar Trafficking of the Mouse Mammary Tumor Virus Gag Protein Induced by Interaction with Ribosomal Protein L9

Darrin V. Bann*, Andrea R. Beyer*, Breanna Rice, Ingrid S. Pultz, Melissa Kane, Stephen P. Goff, Tatyana V. Golovkina, and Leslie J. Parent

*Authors contributed equally


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Abstract

The mouse mammary tumor virus (MMTV) Gag protein directs the assembly in the cytoplasm of immature viral capsids, which subsequently bud from the plasma membranes of infected cells. MMTV Gag localizes to discrete cytoplasmic foci in mouse mammary epithelial cells, consistent with the formation of cytosolic capsids. Unexpectedly, we also observed an accumulation of Gag in the nucleoli of infected cells derived from mammary gland tumors. To detect Gag-interacting proteins that might influence its subcellular localization, a yeast two-hybrid screen was performed. Ribosomal protein L9 (RPL9 or L9), an essential component of the large ribosomal subunit and a putative tumor suppressor, was identified as a Gag binding partner. Overexpression of L9 in cells expressing the MMTV(C3H) provirus resulted in specific, robust accumulation of Gag in nucleoli. Förster resonance energy transfer (FRET) and co-immunoprecipitation analyses demonstrated that Gag and L9 interact within the nucleolus, and the CA domain was the major site of interaction. In addition, the isolated NC domain of Gag localized to the nucleolus, suggesting that it contains a nucleolar localization signal (NoLS). To determine whether L9 plays a role in virus assembly, small interfering RNA (siRNA)-mediated knockdown was performed. Although Gag expression was not reduced with L9 knockdown, virus production was significantly impaired. Thus, our data support the hypothesis that efficient MMTV particle assembly is dependent upon the interaction of Gag and L9 in the nucleoli of infected cells.

*Please see Appendix A for a reprint of the journal article comprising this data chapter.
Chapter 3

Assembly of a Murine Betaretrovirus In Association With Stress Granule-Associated Proteins

Darrin V. Bann, Andrea R. Beyer, and Leslie J. Parent

Abstract

The Gag protein of mouse mammary tumor virus (MMTV) orchestrates the assembly of complete, immature capsids in the cytoplasm of infected cells. The yeast Ty1 and Ty3 retrotransposons follow a morphogenetic pathway similar to MMTV and assemble virus-like particles in cytoplasmic ribonucleoprotein complexes called “retrosomes,” which contain cellular mRNA binding proteins associated with stress granules (SGs) and processing bodies. These observations led us to conduct parallel experiments in MMTV to test whether sites of metazoan retrovirus immature capsid formation also contained SG proteins. We found that cytoplasmic sites of MMTV capsid assembly were distinct from SGs but colocalized with the SG-associated protein YB1, which was required for efficient virus production. Overexpression of YB1 and other SG-associated proteins, including G3BP1, induced the accumulation of Gag within SGs through an RNA-dependent mechanism. To test whether immature particle formation occurred within SGs, we expressed an MMTV Gag-mCherry fusion protein in living mouse mammary cells and used fluorescence recovery after photobleaching (FRAP) analysis to identify early, mid, and late phases of immature capsid assembly. Although we did not observe assembly of MMTV capsids within SGs, recovery of Gag-mCherry fluorescence after photobleaching was significantly reduced in SGs induced by YB1 overexpression compared to SGs induced by G3BP1 overexpression. These results suggest that Gag is stably bound to a factor within YB1-induced SGs, possibly the viral genome. Together, these data suggest a model whereby YB1 recruits viral RNA into cytoplasmic RNPs where it is then bound by Gag for encapsidation into immature intracytoplasmic particles.
3.1 Introduction

Retroviruses are positive sense single-stranded RNA viruses that encode a limited genome and are therefore dependent on host proteins to mediate entry, transcription, translation, and virus release from the cell. However, the host cell employs a number of defense mechanisms to limit or restrict viral replication, which viruses must circumvent or disable to facilitate their own replication. Stress granules (SGs) represent one such defense pathway that restricts the replication of diverse virus families, suggesting they constitute a fundamental regulatory system central to host-pathogen interplay.

SGs are cytoplasmic ribonucleoprotein complexes (RNPs) that form in response to cellular stress including heat shock, oxidative stress, ultraviolet irradiation, and virus infection [257,331,332]. The formation of SGs can be induced by several stimuli, however the classical pathway involves the phosphorylation of the translation initiation factor eIF2α by one of four kinases, including protein kinase R, which recognizes double-stranded viral RNA, and heme-regulated inhibitor (HRI), which responds to oxidative stress and heat shock (reviewed in [333]). Phosphorylation of eIF2α reduces cellular levels of the initiator eIF2-GTP-Met-tRNA complex available for translation initiation, leading to the accumulation of stalled 48S initiation complexes on cellular mRNAs that accumulate in SGs [259,334]. Several proteins with reported antiviral properties localize to SGs, notably Ras-GAP SH3 domain binding protein 1 (G3BP1), apolipoprotein B mRNA editing enzyme 3G (APOBEC3G), argonaute 2 (Ago2), and Y-box binding protein-1 (YB1) [279,287,335,336,337]. As a result, SGs may
interfere with viral replication by inhibiting translation of viral proteins and/or disrupting viral RNA metabolism [287,338].

However, many RNA viruses have evolved to circumvent, manipulate, or commandeer the SG response to promote viral replication. For example, respiratory syncytial virus and Semliki forest virus induce SG formation to shut down cellular protein synthesis during infection while the synthesis of viral proteins is unaffected [308,311]. By contrast, poliovirus inhibits SG formation by cleaving G3BP1 at late times during infection [279,339], whereas hepatitis C virus (HCV) hijacks G3BP1, incorporating it into the viral replication machinery [340,341]. Among retroviruses, both human T-cell leukemia virus type-1 (HTLV-1) and human immunodeficiency virus type-1 (HIV-1) inhibit SG formation [148,314]; however, independently of SG formation, HIV-1 interacts with the SG protein Staufen-1 to facilitate virus production and encapsidation of the viral genome [148,318]. Similarly, the retrovirus-like yeast retrotransposon Ty3 interacts with SG proteins in so-called “retrosomes,” cytoplasmic sites that are distinct from SGs but contain many of the mRNA processing factors typically found in SGs, to promote efficient ribonucleoprotein (RNP) complex formation essential for retrotransposition [170,171]. Given the diversity of viruses and virus-like elements that interact with the SG pathway, manipulating SG components appears to be a critical step for productive replication of many RNA viruses.

Mouse mammary tumor virus (MMTV) is an oncogenic betaretrovirus that differs morphogenetically from HTLV-I and HIV-1 in that complete immature capsids assemble at discrete cytoplasmic sites [342] in a similar fashion as Ty3.
The genesis of MMTV virions is directed by the N-terminally myristoylated Gag polyprotein, which is composed of seven distinct domains: MA or matrix, the plasma membrane-targeting domain [194]; pp21, a phosphoprotein of unknown function; p3, p8, and n, needed for Gag-Gag interactions involved in immature capsid assembly [162,163]; CA or capsid, a major site of Gag multimerization which, upon cleavage of the Gag precursor, forms the mature viral capsid shell; and NC or nucleocapsid, which binds to the viral genomic RNA for encapsidation (reviewed in [343]). Although retroviral Gag proteins are sufficient to assemble virus-like particles in vitro, cellular factors play an important role in virus particle assembly in vivo [342,344,345,346,347,348,349,350]. However, cellular proteins that participate in the assembly of intracytoplasmic MMTV capsids have not been identified, and in particular, it is not known whether SG components are involved in MMTV particle production.

To address this question, we examined whether SG proteins were present at intracytoplasmic sites in association with MMTV Gag proteins. We found that several constituents of SGs colocalized with MMTV Gag in infected cells, and when expressed alone, the Gag itself accumulated in SGs. We found that YB1, a central regulator of cellular mRNA translation [351] that accumulates in SGs, interacted with Gag in an RNA-dependent manner. Moreover, knockdown of YB1 impaired virus production independently of the level of Gag translation. In striking contrast to HIV-1 and HTLV-1, MMTV infection did not inhibit SG formation; rather MMTV-infected cells appeared to be “primed” to form SGs more readily, as overexpression of YB1 led to SG formation at a significantly higher frequency in
infected cells compared to uninfected cells. Taken together, our results suggest that MMTV has evolved to take advantage of the SG pathway by co-opting YB1 to facilitate virus production.

3.2 Materials and Methods

3.2.1 Cell culture, plasmids, and transfections. Uninfected NMuMG (Normal Murine Mammary Gland) cells (ATCC CRL-1636), MMTV-infected NMuMG-C3H cells (a gift of Tatyana Golovkina, University of Chicago) [352,353], and QT6 (quail fibroblast) [354] cells were maintained in culture as previously described [342,355]. Rous sarcoma virus (RSV)-infected QT6 cells were established by transfection of the pRC.V8 molecular clone [355]. Plasmids expressing myc-YB1, myc-HuR, and myc-PABPc1 were gifts from Michael Malim (Kings College, London) [337]. The YFP-TIA-1 plasmid was a gift from Nancy Kedersha (Harvard University) [337] and pGFP-G3BP1 was a gift from Jamal Tazi (where) [271]. The MMTV pGag-GFP construct was described [356].

The pGagΔNC-mCherry plasmid was created by PCR amplifying the mCherry gene and replacing the GFP sequence in pMA-CA.Gag-GFP [342] using BamHI-NotI. The CREB1 leucine zipper domain coding sequence was PCR amplified from RSV pGag.Zip-GFP [185] and inserted into pGagΔNC-mCherry using EcoRI-BamHI to create pGag.Zip-mCherry. To create the pGag.pp21.imCherry construct, we first created pGag-4xCTE by inserting MMTV Gag in place of HIV-1 Gag in pGPV-4xCTE [357] (a kind gift from Michael Malim, Kings College, London) using EcoRI-BamHI. The PflMI site within mCherry was deleted through a silent mutation using site-directed mutagenesis and the
resultant mCherry gene was inserted between the seventh and eighth codons of pp21 using splicing by overlap extension [358]. The resulting fragment was then inserted into pGag-4xCTE using Sacl-PflMI sites present within MMTV Gag. PCR primer sequences are available upon request. NMuMG cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions whereas QT6 cells were transfected using the calcium phosphate method [355].

3.2.2 Immunofluorescence and confocal microscopy

At various times following transfection, cells were washed twice with phosphate buffered saline (PBS), fixed in 3.7% paraformaldehyde, permeabilized in 0.25% Triton-X100, and blocked in 10% bovine serum albumin. Samples were immunostained using rabbit α-myc (AbCam Ab9106), rabbit α-YB1 (AbCam Ab12148), rabbit α-TIA1 (AbCam Ab40693), and/or mouse monoclonal anti-CA (a gift from Tatyana Golovkina) primary antibodies followed by appropriate anti-rabbit-Cy3 (Sigma C2306), anti-rabbit-Cy5 (AbCam Ab97077), anti-mouse-Cy3 (Sigma C2818), or anti-mouse-FITC (Sigma F9137) secondary antibodies. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (EMD Chemicals, Inc.) and mounted on glass slides using SlowFade reagent (Invitrogen).

Samples were imaged using a Leica TCS SP2-ABOS confocal microscope (Leica Microsystems, Wetzlar, Germany). Images were obtained using sequential scanning with laser excitations of 405 nm (DAPI), 488 nm (eGFP), 514 nm (YFP), 543 nm (Cy3), or 633 nm (Cy5). Colocalization analysis was conducted using Colocalization Colormap [359] and/or Just Another Colocalization Plugin [360] for Image J [361]. Thresholds were set to approximately the same levels of
fluorescence present in the original confocal image. Images were prepared for publication using CorelDrawX3 (Corel Corp., Ottawa, Canada). Any adjustments to intensity were applied to all images in a series.

### 3.2.3 Fluorescence recovery after photobleaching (FRAP)

Cells were seeded onto 35 mm glass bottom dishes (MatTek Corp.), transfected as above, and imaged 15 – 18 hours after transfection using a Leica TCS SP8-ABOS confocal microscope fitted with a 37°C, 5% CO₂ live cell chamber. Samples were imaged using a white light laser tuned to 489 nm for GFP or 580 nm for mCherry. Cells were imaged for approximately 20 seconds (pre-bleach), then two circular, 5 µm diameter regions of interest (ROIs) per cell were subjected to photobleaching for approximately 10 seconds using the 580 nm white light laser at 100% power. Cells were then monitored for 15 minutes after bleaching.

Individual foci were selected for analysis in ImageJ by drawing 1.5 µm diameter ROIs around foci and monitoring the integrated signal intensity over the course of the experiment. To ensure that foci were bleached to at least 30% of the initial fluorescence intensity, data were first normalized by the method of Phair et al. [362] using the following formula in Microsoft Excel:

\[
I_{\text{Phair}}(\text{AU}) = \frac{AC_{\text{pre}}}{AC_{t-BG_t}} \times \frac{I_{\text{pre}}}{I_{t-BG_t}} \text{ where } I_{\text{Phair}} \text{ is the normalized relative intensity (in arbitrary units) at time } t; \ AC_{\text{pre}} \text{ is the average total cell pre-bleach intensity; } AC_t \text{ is the total cell fluorescence intensity at time } t; \ BG_t \text{ is the background fluorescence intensity at time } t; \ I_{\text{pre}} \text{ is the average experimental ROI pre-bleach intensity; and}
\]

70
$I_t$ is the experimental ROI fluorescence intensity at time $t$. Foci that were not bleached to $\leq 30\%$ of the normalized initial intensity were discarded from the analysis. To facilitate fitting of the data by GraphPad Prism, the data were subjected to additional full-scale normalization [363] using the following formula:

$$I_{FS_t}(AU) = \frac{I_{Phair} - I_{Phair0}}{I_{Phair0}}$$

where $I_{FS_t}$ is the full-scale normalized value at time $t$ and $I_{Phair0}$ is the first post-bleach value. Average signal intensities and standard errors of the mean for each timepoint from at least four foci were calculated using Microsoft Excel and fit using single or double exponential association curves using GraphPad Prism.

### 3.2.4 Immunoelectron Microscopy

To prepare samples for transmission electron microscopy, cells were seeded into 60-mm dishes containing one 22-mm Thermanox coverslip (Thermo Scientific), which was processed for electron microscopy, and one standard glass coverslip, which was processed for immunofluorescence as described above. To induce SG formation, cells were transfected with GFP-G3BP1 for 18 hours or were subjected to heat shock at 44°C for 45 minutes. To prepare thermanox coverslips for electron microscopy, cells were fixed in 3.7% paraformaldehyde plus 0.2% gluteraldehyde, permeabilized with 0.25% Triton X-100, and blocked in 10% BSA. Samples were then incubated with rabbit $\alpha$-YB1 or rabbit $\alpha$-GFP (a gift from John Wills, Penn State College of Medicine), followed by a 1.4nm gold-conjugated goat $\alpha$-rabbit secondary antibody (Nanoprobes, Inc. #2003). Samples were subsequently post-fixed in 4% paraformaldehyde plus 0.5% gluteraldehyde
for 1 hour at room temperature and nanogold particles were enhanced using HQ Silver reagent (Nanoprobes, Inc #2012). Cells were prepared for embedding by incubating in 0.5% osmium tetroxide for 1 hour, followed by ethanol dehydration and embedding in EMbed812 epoxy resin (Electron Microscopy Sciences). 70 – 90 nm thin sections were cut using a diamond knife mounted on a Porter-Blum MT-2B microtome and placed on 200 mesh copper grids. Sections were stained with 2% aqueous uranyl acetate followed by lead citrate and imaged on a JEOL JEM 1400 transmission electron microscope with an Orius SC1000 bottom mounted CCD camera.

3.2.5 Co-immunoprecipitations

Co-immunoprecipitations (co-IPs) were performed essentially as described [356], with the following changes. Cells were lysed in 50 mm Tris pH 7.05, 1% Triton-X100, 0.5% deoxycholic acid, 150 mM NaCl on ice for 15 minutes, cellular debris was removed by centrifugation, and half of each lysate was treated with 25 µg RNaseA (Invotrogen) for 15 minutes. Rabbit α-YB1, rabbit α-G3BP1 (Bethyl Laboratories) #, or rabbit anti-Lamin A/C (Santa Cruz Biotechnology #) antibodies were added to cleared lysates for 1 hour at room temperature. Protein A sepharose beads (Invitrogen) were washed twice in coIP buffer (50 mM Tris pH 8.0, 1% Triton-X100, 0.1% SDS, 1% deoxycholic acid, and 150 mM NaCl), added to each lysate sample, and incubated overnight at 4°C with gentle end-over-end mixing. After pelleting by centrifugation, beads were washed twice with Co-IP buffer and twice with TE buffer (10 mM Tris-HCL pH 7.5, 1 mM EDTA pH 8.0), resuspended in SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 20%
glycerol, 0.5% bromophenol blue, 4% SDS, and 10% β-mercaptoethanol), and boiled. Eluted proteins were separated by SDS-PAGE and transferred to PVDF membranes (BIORAD). Western blotting was performed using α-CA antibody at 1:X dilution followed by goat α-mouse-horseradish peroxidase conjugate antibody diluted to 1:10,000. Chemiluminescent signals were developed using SuperSignal® West Pico Substrate (Thermo Scientific).

3.2.6 siRNA Knockdowns

YB1 or TIA1 expression was knocked down in NMuMG.C3H cells by transfection with 5 pmol of YB1-specific siRNAs (5’ CUGAGUAAUGCCGCUUA[dT][dT], 5’ CGAUCCACCAGCUGAGAAU[dT][dT]; Sigma-Aldrich) or TIA1-specific siRNA (5’ CAGCACACAGCGUUCACAA[dT][dT]; Sigma-Aldrich) for 16 hours using Lipofectamine 2000. Control cells were transfected with scrambled siRNA (siRNA Universal Negative Control #1 [SIC001]; Sigma-Aldrich). Transfection media was replaced by primary growth media for 8 hours and cells were transfected with an additional 5 pmol of YB1, TIA-1 or control siRNA. To replete YB1 expression, cells were transfected with 2µg of siRNA-resistant pmyc-YB1 during the initial siRNA transfection.

3.2.7 Virus Budding Assays

The MMTV budding assay was described previously [356]. Briefly, MMTV-infected NMuMG.C3H cells were transfected with pYFP-TIA1, p.EGFP.N2 (mock), YB1-specific siRNA, TIA1-specific siRNA, or control siRNA, as above. One day after transfection, virus particles released into the medium were collected for 3 hours, pelleted through a 20% sucrose cushion, and resuspended in SDS-PAGE
sample buffer. Proteins present in the corresponding cell lysates and supernatants were separated by SDS-PAGE and the Gag/CA protein was detected by Western blotting using anti-CA antibody with quantitative imaging of chemiluminescence using Ultraviolet Products EC3 Chemi HR 410 imaging system (UVP; Upland, CA). Virus budding efficiency was calculated as the amount of CA protein detected in the supernatant (virus, V) divided by the sum of the Gag/CA protein present in the lysate (L) and supernatant using the equation $V / (V+L)$ [356]. Expression levels of YB1 and TIA1 (endogenous and epitope-tagged proteins), as determined by Western blotting using α-YB1 or α-TIA1 antibodies, were compared to GAPDH loading controls using goat α-GAPDH antibody (GenScript #). Western blots were developed using Amersham ECL-Plus reagent (GE Healthcare Life Sciences) or SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) and quantitated using the UVP imaging system described above. Budding assays were performed at least 8 times in separate transfections conducted on 2 different days. The mean, standard error of the mean, and $p$-values were calculated using two-tailed paired Student’s t tests (GraphPad Prism 5, GraphPad Software, Inc).

3.3 Results

3.3.1 MMTV colocalizes with YB1, but not TIA1, in non-stressed cells.

Previous studies of HIV-1 and HTLV-I, retroviruses following the C-type morphogenetic pathway and assembling capsids at the plasma membrane, reported that SG formation was inhibited by infection with these viruses. By
contrast, the yeast retrotransposon Ty3 assembles Gag-RNA RNP complexes in the cytoplasm in conjunction with a number of mRNA processing factors that include orthologs of mammalian SG proteins. Therefore, we reasoned that MMTV may take advantage of a similar set of host factors as Ty3, since MMTV assembles viral RNPs and immature capsids at distinct intracytoplasmic sites. To test this possibility, we began our studies with two constituents of SGs, YB1 and TIA1, because antibodies were available that could reliably detect these endogenous proteins in mouse cells.

YB1 is a major component of cellular messenger ribonucleoprotein complexes (mRNPs) that accumulates in SGs following cellular stress, and it serves as a key translational regulator in mammalian cells. YB1 is essential for viability in mice, but it does not have a homologue in *Saccharomyces cerevisiae* so its role in Ty3 assembly has not been examined [364,365]. Moreover, YB1 is not considered a core component of SGs, and overexpression of YB1 does not initiate SG formation. On the other hand, TIA1 is a core scaffold SG protein and its overexpression is sufficient to form cytoplasmic RNPs with properties of authentic SGs. TIA1 is a nucleocytoplasmic shuttling protein that redistributes from the nucleus to the cytoplasm and recruits mRNAs to SGs during stress [274].

To examine whether MMTV cytoplasmic RNPs colocalize with YB1 or TIA1 under steady-state conditions, NMuMG cells were immunostained with YB1 or TIA1 antibodies and examined using deconvolution microscopy. Initially, we assessed the distributions of these proteins in uninfected NMuMG cells. YB1 had a speckled cytoplasmic distribution, characteristic of its localization within cellular
Figure 3.1. MMTV Gag colocalizes with endogenous YB1. A) Uninfected NMuMG cells were unstressed, subjected to heat shock at 44°C for 45 minutes (HS), or heat shocked for 30 minutes at 44°C and treated with cyclohexamide (HS + CHX) or vehicle control (HS + DMSO) for 15 minutes at 44°C, then fixed and stained for YB1. B) Uninfected NMuMG cells were treated as in (A) and immunostained for TIA1. C) MMTV(C3H)-infected NMuMG cells were treated as above and immunostained for YB1 and Gag. Boxes indicate the area of enlargement. M₁ and M₂ coefficients are shown in the upper right-hand corner, and were calculated for ≥6 cells using Just Another Colocalization Plugin (JACoP) [360] for ImageJ [361]. Values are shown as the mean ± standard error of the mean. C) MMTV(C3H) cells treated as above and immunostained for TIA1 and Gag. M₁ and M₂ values were calculated for 10 cells in each condition.
mRNPs (Fig. 3.1A, unstressed) [270,364], whereas TIA1 was predominantly nuclear (Fig. 1B, unstressed), with a slightly speckled cytoplasmic appearance. When these cells were subjected to heat shock at 44°C for 45 minutes, YB1 and TIA1 accumulated in SGs, large cytoplasmic aggregates, as previously reported in other cell types (Fig. 3.1A and 3.1B, HS). SGs formation depends on the presence of non-translating mRNAs, which interchange dynamically between SGs and polysomes [289]. Thus, treating stressed cells with the translational inhibitor cyclohexamide (CHX) causes SGs to disassemble despite continued stress [365]. Indeed, when NMuMG cells were heat shocked for 30' at 44°C, then treated with CHX for an additional 15' at 44°C, the distributions of YB1 and TIA1 were similar to those unstressed NMuMG cells, indicating that CHX treatment caused SGs disassembly (Fig. 3.1A and 3.1B, HS + CHX). By contrast, cells subjected to the same conditions but treated with vehicle control (DMSO), YB1 and TIA1 still accumulated within large SGs (Fig. 3.1A and 3.1B, HS + DMSO).

We next examined the distribution of endogenous YB1 and TIA1 in NMuMG cells chronically infected with the viral strain MMTV.C3H cells, and MMTV capsids were stained using anti-CA antibody. In unstressed cells, a subpopulation of YB1 colocalized with a portion of cytoplasmic Gag foci, suggesting that YB1 partially localizes to sites of MMTV capsid assembly (Fig. 3.1C, unstressed). Overlap between Gag and YB1 immunofluorescence was quantitatively assessed using Mander's colocalization analysis [366], which revealed 16% overlap of YB1 with Gag (M; 0.16 ± 0.03) (and 35%
colocalization of Gag with Yb1 ($M_2 = 0.35 \pm 0.03; n = 10$). These data indicate that Gag colocalizes with a population of YB1 in small cytoplasmic granules. By contrast, Gag and TIA1 colocalization was much less common under identical conditions ($M_1 = 0.02 \pm 0.00; M_2 = 0.09 \pm 0.02; n=10$; Fig. 3.1D), suggesting that TIA1 is not present at MMTV sites of capsid assembly in unstressed cells.

We next tested whether Gag would accumulate in SGs by heat shocking MMTV.C3H-infected cells for 45' at 44°C. During HS, a fraction of YB1 accumulated in large SGs and, surprisingly, Gag was also present in SGs (Fig. 3.1C, HS, arrow). Interestingly, the same degree of endogenous YB1 protein remained associated with Gag in small cytoplasmic foci without significantly altering the $M_1 (0.16 \pm 0.03)$ or $M_2 (0.40 \pm 0.05)$ coefficients ($n = 10$, $p= X$)). Similarly, heat shock did not change the degree of colocalization between Gag and TIA1 ($M_1 = 0.03 \pm 0.01; M_2 = 0.11 \pm 0.01; n=10$), even though a small amount of Gag also accumulated in TIA1-containing SGs (Fig. 3.1D, arrow). Additionally, we noted an increase in the Gag signal in the nucleus during HS (Fig. 3.1C, and D). We previously reported that a subpopulation of Gag localizes to the nucleus and nucleolus under steady-state conditions [342], however the significance of Gag localization to the nucleus during HS is not known.

Treating cells with CHX during heat shock dissolved SGs, although a population of YB1 remained associated with Gag foci (Fig. 3.1C, HS + CHX) without any change in the $M_1 (0.16 \pm 0.03)$ and $M_2 (0.29 \pm 0.06)$ coefficients ($n=10$). Similarly, CHX treatment did not alter colocalization of Gag and TIA1 (Fig. 1D) ($M_1 = 0.02 \pm 0.00; M_2 = 0.11 \pm 0.01; n=10$). These experiments
demonstrated that MMTV infection, by contrast to HIV-1 and HTLV-I, does not suppress the formation of SGs [148,314]. Moreover, MMTV Gag is associated specifically with the SG-associated protein YB1 in small cytoplasmic granules, which are distinct from heat shock-induced SGs and insensitive to translational inhibition by CHX treatment. Interestingly, Gag also accumulated in HS-induced SGs, which were sensitive to CHX, suggesting that Gag may transiently localize to SGs during cellular stress, possibly due to its association with YB1.

3.3.2 Overexpression of additional SG proteins causes accumulation of Gag in SGs in MMTV-infected cells.

Cultured cells only tolerate HS for relatively short periods of time [270], therefore to determine whether a larger proportion of MMTV Gag proteins would move into SGs over a longer period of time, we induced SG formation by transient overexpression of several epitope-tagged SG-associated proteins [365]. TIA1, G3BP1, and tristetraproline (TTP) serve a scaffolding role in SGs, and overexpression of these proteins reliably causes the nucleation of large cytoplasmic aggregates that disassemble with CHX treatment, similarly to authentic SGs [271,272,273]. As expected, expression of GFP-G3BP1 and YFP-TIA1 resulted in the formation of numerous large SGs, whereas SGs formed by TTP-myc overexpression tended to be smaller in size and fewer in number (Fig. 3.2B). MMTV Gag accumulated to a high degree in SGs induced by GFP-G3BP1 expression, with Mander's analysis revealing that 95% of GFP-G3BP overlapped with Gag ($M_1 0.95 \pm 0.02$) while approximately 69% of Gag colocalized
Figure 3.2. Expression of SG-associated proteins relocalizes MMTV Gag to SGs. Confocal microscopy images showing MMTV Gag localization with myc-YB1 (A), proteins that nucleate SG assembly (B), and proteins that localize to SGs but have not been reported to nucleate SG assembly (C). Colocalization colormaps were generated using the Colocalization Colormap plugin [359] for ImageJ [361]. Boxes indicate the area shown in “Enlargement”. 
with GFP-G3BP1 ($M_2 0.69 \pm 0.06; n=10$). In YFP-TIA1 expressing cells, ~64% of Gag overlapped with YFP-TIA1 ($M_2 0.64 \pm 0.05$) and a smaller fraction of YFP-TIA1 overlapped with Gag ($M_1 0.38 \pm 0.07, n=10$). There was a similar amount of colocalization between TTP-myc with Gag and vice versa ($M_1 0.51 \pm 0.07$ and $M_2 0.37 \pm 0.04, n=10$).

Although Mander’s colocalization analysis quantifies the amount of each fluorophore present within the same pixel as a comparator fluorophore in the same image, we used colocalization colormapping [359] [361] to determine whether the fluorescence intensities of the tagged proteins were similar to one another within coclocalized areas. This ImageJ algorithm uses thermal pseudocoloring to plot normalized mean deviation product (nMDP) colocalization coefficients on a map of the original image, where red-orange colors indicate areas of high signal correlation, and blue-green colors indicate areas of low correlation between channels [359]. This analysis revealed a high correlation (red-orange color) between Gag and G3BP1, TIA1, and TTP within SGs (Fig. 3.2B, box), indicating that the local concentrations of both Gag and each SG constituent was very high.

To examine whether MMTV Gag also accumulated in SGs when SG-associated proteins that do not directly nucleate SG formation were overexpressed, we expressed myc-YB1, myc-HuR and myc-PABPc1 in NMuMG.C3H cells [257,267,275]. Interestingly, overexpression of each of these proteins induced SG formation, and Gag was accumulated to a high degree in these SGs (Fig. 3.2C). In cells containing YB1-induced SGs, MMTV Gag was
relocalized from small cytoplasmic foci to large SGs where it colocalized with YB1 \((M_1=0.26 \pm 0.02; M_2=0.44 \pm 0.03; n=10; \text{Fig. 3.2A})\). Similarly, the \(M_1\) colocalization coefficient for myc-HuR with Gag was 0.25 \(\pm 0.05\) and the \(M_2\) value for Gag with myc-HuR was 0.55 \(\pm 0.06\). Similarly, \(\sim 25\% \ (M_1 0.25 \pm 0.03)\) of myc-PABPc1 overlapped with Gag and the \(M_2\) value was 0.53 \(\pm 0.04\), indicating that expression of myc-PABPc1 caused a similar proportion of Gag to accumulate in SGs. For mycYB1, myc-HuR and myc-PABPc1, colocalization colormapping revealed that Gag accumulated in SGs with these proteins at high concentration (Fig. 3.2C, boxes).

To discern whether Gag accumulation in SGs was specific to overexpression of SG-associated proteins, we transfected NMuMG cells with YFP-tagged trans-Golgi network 38 (TGN38), an integral membrane protein that cycles between the Golgi and the plasma membrane [367]. Gag partially overlapped with TGN38-YFP, as indicated by Mander’s analysis \((M_1=0.08 \pm 0.01; M_2=0.15 \pm 0.05)\) and colocalization colormapping (Fig. 3.2D), however the distribution of Gag in cells expressing TGN38-YFP was grossly unaffected and Gag did not accumulate in large granules. As a whole, these data indicated that inducing SG formation by overexpressing SG-associated proteins dramatically alters the subcellular distribution of MMTV Gag and causes Gag to accumulate in SGs.
3.3.3 Gag accumulates in SGs over time

To this point, our data indicated that Gag accumulated in SGs induced by chronic stimuli (i.e. overexpression), but not acute stimuli such as HS. Because overexpression of SG proteins and HS both induce SG formation through a similar pathway (i.e. phosphorylation of eIF2α) [269], these results suggest a time-dependent accumulation of Gag in SGs. To test this hypothesis, we visualized the distribution of Gag in MMTV-infected NMuMG-C3H cells at various timepoints following transfection with GFP-G3BP1.

Immediately after transfection Gag had a punctate cytoplasmic distribution and no GFP-G3BP1 was detectable. Accordingly, colocalization analysis revealed no overlap between GFP-G3BP1 and Gag signals (Fig. 3.3A, B). By six hours after transfection, GFP-G3BP1-containing SGs were readily apparent, although there was relatively little overlap between GFP-G3BP1 and Gag (M1 =0.28 ± 0.10; M2 = 0.19 ± 0.06; n=10) (Fig. 3.3A, B). However, colormapping indicated a correlation between Gag and G3BP1 within SGs (Fig. 3.3A, arrows). Strikingly, 3D analysis revealed that small Gag foci were present within or on the edge of G3BP1-containing SGs, suggesting a dynamic interaction between MMTV Gag foci and SGs (data not shown). After 12 hours, the overlap between G3BP1 and Gag was relatively unchanged, however a significantly greater proportion of Gag overlapped with G3BP1 (M1 =0.38 ± 0.04, p>0.05; M2 =0.37 ± 0.06, p<0.05; n=10) (Fig. 3.3A, B). Furthermore, colormapping indicated a higher correlation between the Gag and G3BP1 signals within SGs, indicating that Gag was
**Figure 3.3. MMTV Gag accumulates in SGs over time.** A) MMTV(C3H)-infected NMuMG cells were transfected with GFP-G3BP1, fixed at various times post transfection, and immunostained for Gag. Average M1 and M2 values are shown in the upper right-hand corner of GFP-G3BP1 and α-CA/Gag images, respectively. Colocalization colormaps are shown on the right. B) Graphs showing average M1 and M2 values at each timepoint. Error bars indicate standard error of the mean.
accumulating within SGs (Fig. 3.3A, arrows). At 24 hours post-transfection, there were significant increases in both the proportion of G3BP1 overlapping with Gag and vice versa ($M_1 = 0.85 \pm 0.06, p<0.005; M_2 = 0.71 \pm 0.06, p<0.005$) and colocalization colormapping indicated a strong correlation between the Gag and G3BP1 signals within SGs (Fig. 3.3A, B). As a result, our data support a model in which Gag accumulates in SGs in a time-dependent manner.

### 3.3.4 Functional role of SG proteins in MMTV capsid assembly

Because retroviral RNAs are analogous to cellular mRNAs, and YB1 has been found to either restrict or promote replication of Dengue virus and Hepatitis C virus, respectively [287,368], we tested whether YB1 plays a functional role in MMTV replication. To do this, we knocked down YB1 using siRNAs and measured the effect on MMTV virus production by NMuMG.C3H cells. Although YB1 knockout is embryonic-lethal [369], we were able to reduce YB1 expression by $77 \pm 7\%$ relative to scrambled control siRNAs ($p=0.04$) (Fig. 3.4A). The reduction in YB1 expression led to a $58 \pm 7\%$ reduction in virus particle production relative to scrambled controls ($p=0.02$). This finding is striking considering every cell is MMTV-infected but the YB1 siRNA was delivered by transient transfection and is therefore likely to reduce YB1 expression only in a portion of cells. To test whether the observed budding defect was specific to YB1, we performed a rescue experiment by co-transfecting cells with YB1 siRNA and an siRNA-resistant myc-YB1 expression plasmid. Expression of the myc-YB1 plasmid returned cellular...
Figure 3.4. Functional role of YB1 in MMTV assembly. A) Graph showing mean YB1 expression in NMuMG-C3H cells treated with YB1 siRNA relative to cells treated with scrambled siRNA control. YB1 expression was determined by Western blotting and standardized to GAPDH loading controls. A representative Western blot is shown to the right. B) Graph depicting mean virus production from experiments described in (A). Virus production was determined by Western blotting and is calculated as signal from pelleted virus over the signal from pelleted virus plus signal from whole-cell lysate (V/V+L) [342]. A representative Western blot is shown to the right of the graph. C) Relative TIA1 expression and (D) virus production from NMuMG-C3H cells treated with TIA1 siRNA or control siRNA. Western blots from representative experiments are shown to the right. E) Overexpression of TIA1 and (F) the effect on virus production relative to cells transfected with a GFP control plasmid. Representative Western blots are shown to the right of each graph. All graphs show the mean ± standard error of the mean. Statistical significance (* p<0.05) was determined by Student’s T-test (Graph Pad Prism).
YB1 levels to 66 ± 5% of cells treated with control siRNA and rescued virus production to 84 ± 16% (p=0.60) of scrambled siRNA controls.

Our finding that modulating YB1 expression reduced virus production next led us to test whether other SG-proteins also influenced virus production. To this end, we used siRNA to knock down expression of TIA1 by approximately 80% relative to cells treated with scrambled control siRNAs (Fig. 3.4B). Interestingly, depletion of TIA1 had no significant effect on virus production (Fig. 3.4B), although the TIA1-like protein, TIAR, which was unaffected by TIA1 knockdown, may be able to compensate for the depletion of TIA1. Furthermore, when TIA1 was overexpressed by 200-fold relative to mock-transfected cells, virus production was also unaffected (Fig. 3.4C). Similarly, overexpression of G3BP1 also did not affect virus production (data not show). As a result, we concluded that MMTV assembly pathway interacts with YB1 specifically to promote capsid assembly.

### 3.3.5 Gag is sufficient for SG localization in the absence of other viral proteins

To understand why Gag might localize to SGs, we conducted experiments to determine the mechanism by which Gag interacts with SG proteins. First, we tested whether Gag was sufficient to traffic to SGs by co-expressing fluorophore-tagged Gag (Fig. 3.5A) with myc-YB1, GFP-G3BP1, or YFP-TIA1. Interestingly, in NMuMG cells expressing myc-YB1 and Gag-GFP, we did not observe large SGs and myc-YB1 instead was diffusely cytoplasmic with occasional small foci.
Figure 3.5. Gag is sufficient to traffic to SGs. GFP- or mCherry-labeled MMTV Gag [342] was coexpressed with myc-YB1 (A), SG-nucleating proteins (B), or non-nucleating SG-associated proteins (C) in uninfected NMuMG cells. Colocalization colormaps were generated as above and are shown under “Colocalization”.
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In fact, expression of myc-YB1 in the absence of MMTV infection induced the formation of SGs in only 6.3% of NMuMG cells (n=190), compared to 31.6% of MMTV-infected NMuMG cells (p<0.0001) (Fig. 3.S1). However, retroviral infection in general does not promote the formation of SGs in response to YB1 overexpression, as there was no difference in the percentage of cells containing SGs when YB1 was expressed in uninfected (11.4%; n=44) or Rous sarcoma virus-infected (0.0%; n=34) QT6 cells (p>0.05) (Fig. 3.S1). However, in uninfected NMuMG cells expressing myc-YB1, Gag-GFP formed small foci that did partially colocalize with myc-YB1, although colocalization colormapping did not indicate strong accumulation of Gag within YB1-containing granules (Fig. 3.5B).

In contrast, SGs were observed in NMuMG cells expressing GFP-G3BP1 and Gag-mCherry, and Gag was strongly localized to G3BP1-containing SGs (Fig. 3.5B). Furthermore, in cells expressing YFP-TIA1, Gag also accumulated in TIA1-containing SGs and similar results were observed with overexpression of TTP-myc (Fig. 3.5B). Similarly, myc-HuR or myc-PABPc1 induced SG formation and Gag-GFP accumulated in SGs (Fig. 3.5C). As a result, we conclude that Gag is sufficient to localize to SGs in general, however in the case of YB1, viral infection may promote the formation of SGs in response to YB1 overexpression.

3.3.6 RNA-dependent interactions between Gag and SG-associated proteins

Because SGs are RNA-dependent and Gag is an RNA-binding protein, next tested whether RNA is required for Gag to complex with SG components by conducting co-immunoprecipitation (co-IP) experiments in the absence or
**Figure 3.S1. Effect of MMTV infection on SG formation induced by YB1 overexpression.** A) Uninfected or MMTV-infected NMuMG cells were transfected with myc-YB1 and processed for immunofluorescence 18 hours after transfection, and cells were counted visually to determine the proportion of SG-containing cells. B) In parallel, uninfected or RSV-infected QT6 cells were transfected with myc-YB1 (uninfected) or myc-YB1 and Gag-GFP (RSV-infected). Statistical significance was determined using Fisher's exact test (GraphPad Prism).
presence of RNase A. We first conducted co-IPs in MMTV-infected NMuMG-C3H cells using an antibody to endogenous YB1 (Fig. 3.6A). MMTV Gag is 77 kDa and undergoes extensive intracellular processing before particle release [112,219]. Unprocessed Gag and its cleavage products were detected in both the input lane and in the unbound fractions from the co-IPs (Fig. 3.6A). Additionally, Gag, but not Gag cleavage products, co-immunoprecipitated with YB1. However, treating samples with 25 µg of RNase A prior to conducting the co-immunoprecipitation disrupted the Gag-YB1 interaction, indicating that the Gag-YB1 interaction is RNA-dependent. Gag did not co-immunoprecipitate with a non-specific antibody (IgG) (Fig. 3.6A).

We next examined whether Gag interacts with other SG-associated proteins. Gag also co-immunoprecipitated with endogenous G3BP1, however this interaction appeared to be less robust than we observed with YB1. However, like YB1, the interaction between G3BP1 and Gag was disrupted by RNase treatment (Fig. 3.6B) and only full-length, unprocessed Gag co-immunoprecipitated with G3BP1. Together these data indicate that Gag interacts with SG-associated proteins through an RNA intermediate likely to be the viral RNA.
Figure 3.6. RNA-dependent interaction of Gag with SG proteins. Endogenous YB1 (A) or G3BP1 (B) was immunoprecipitated from MMTV(C3H)-infected NMuMG whole cell lysates. Prior to the immunoprecipitation, some samples were treated with RNase A. Gag (arrow) was detected by Western blotting using mouse anti-CA antibody. C) Diagram depicting the NC deletion mutant Gag.ΔNC-mCherry. D) Gag.ΔNC-mCherry was expressed alone or co-expressed with myc-YB1, GFP-G3BP1, or YFP-TIA1 in uninfected NMuMG cells. Colocalization colormaps were generated as above and are shown to the right. E) Diagram depicting Gag.Zip-mCherry, a mutant in which the NC domain of Gag has been replaced with the CREB1 leucine zipper domain. F) Gag.Zip-mCherry was expressed alone, or co-expressed with myc-YB1, GFP-G3BP1, or YFP-TIA1 in NMuMG cells. Colocalization colormaps are shown to the right.
3.3.7 NC-dependent trafficking of Gag to SGs

The NC domain of Gag is required for both Gag-RNA and Gag-Gag interactions, and deletion of NC results in a diffuse cytoplasmic distribution of Gag [356]. To determine whether NC is required for Gag to localize to SGs, we co-expressed an mCherry tagged Gag truncation mutant where the NC domain was deleted (Gag.ΔNC-mCherry) (Fig. 3.6C) with myc-YB1, GFP-G3BP1, or YFP-TIA1 in NMuMG cells. When expressed alone, Gag.ΔNC-mCherry was diffusely cytoplasmic (Fig. 3.6C) and its distribution was not altered by expression of myc-YB1, GFP-G3BP1, or YFP-TIA1 (Fig. 3.6D), indicating that NC is required for Gag to traffic to NC.

To test whether Gag-Gag or Gag-RNA interactions target Gag to SGs, we inserted the CREB1 leucine zipper domain in place of NC to make Gag.Zip-mCherry (Fig. 3.6E). For HIV-1 and RSV, substitution of the CREB1 zip domain restores Gag-Gag interactions and is sufficient for virus-like particle production [185,186]. Similarly, MMTV Gag.Zip-mCherry formed small cytoplasmic foci in uninfected NMuMG cells (Fig. 3.6E). However, when Gag.Zip-mCherry was co-expressed with myc-YB1, YFP-TIA1, or GFP-G3BP1, the foci formed by Gag.Zip were distinct from SGs (Fig. 3.6E). As a result, we conclude that NC mediates the localization of Gag to SGs, either through a Gag-RNA interaction, or through a signaling motif present within NC.
3.3.8 Dynamic trafficking of MMTV Gag within SGs

The observation that MMTV Gag accumulates in SGs raised the possibility that assembly of MMTV virus particles occurs in SGs induced by the overexpression of SG-associated proteins. To test this possibility, first attempted to visualize assembled MMTV virus particles within SGs induced by heat shock or GFP-G3BP1 overexpression using immunoelectron microscopy. Immunofluorescence analysis confirmed that both treatments induced SG formation, however we did not observe immature virus particles within YB1- or GFP-G3BP1-containing SGs (Fig. 3.S2). This finding suggested that MMTV particle assembly does not occur within SGs, but to formally test this conclusion we developed a fluorescence recovery after photobleaching (FRAP) assay to monitor virus particle assembly in live, MMTV-infected NMuMG cells.

MMTV Gag was fluorescently labeled by inserting mCherry between the seventh and eighth codons of the pp21 domain (Fig. 3.7A), and the resulting Gag.pp21.imCh protein is correctly processed by MMTV protease and is incorporated into virus particles when expressed in MMTV-infected NMuMG.C3H cells (data not shown). Imaging live NMuMG.C3H cells expressing Gag.pp21.imCh revealed that Gag localized to small cytoplasmic foci as expected (Fig. 3.7A). Individual foci were chosen for analysis (Fig. 3.7A, green and red circles) and monitored for approximately 20 seconds, subjected to photobleaching, then monitored for fluorescence recovery for 15 minutes, which encompasses the time frame required for assembly of HIV-1, EIAV, and MLV
Figure 3.S2. Association of MMTV virus particles with SG proteins by immuno-electron microscopy. A) MMTV-infected NMuMG cells were heat shocked at 44°C for 45' and prepared for immune-electron microscopy by staining with α-YB1 antibody (left), or for immunofluorescence using α-YB1 and α-CA/Gag primary antibodies (right). Black arrows show YB1 labeling; black arrowheads indicate complete, immature particles; and grey arrowheads indicate incomplete particles. B) SGs were induced by expressing GFP-G3BP1 in NMuMG-C3H cells. Samples were processed as above using an α-GFP antibody to detect GFP-G3BP1 for immuno-electron microscopy (left) or an α-CA/Gag antibody for immunofluorescence. Black arrows indicate GFP-G3BP1 labeling and black arrowheads indicate complete, immature particles.
A

HS: 44°C, 45

α-YB1

Gag/CA α-YB1

B

GFP-G3BP1

α-GFP

Gag/CA GFP-G3BP1
particles [370,371,372,373]. The fluorescence intensity for individual foci were measured, normalized, and plotted as described in Materials and Methods. We predicted that completely assembled virus particles would exhibit limited fluorescence recovery (Fig. 3.7A, green line), while fluorescence would recover rapidly in cytoplasmic sites containing high local concentrations of Gag that had not yet assembled into higher-order complexes (Fig. 3.7A, red line).

Analysis of 19 individual foci revealed the presence of three populations of Gag foci with distinct kinetics. On average, the first population of foci (n=4) exhibited rapid, two-phase recovery with a fast-phase $t_{1/2}$ of 7.8 seconds (95% confidence interval [CI] 6.7 – 9.3 seconds) and a slow-phase $t_{1/2}$ of 84.2 seconds (95% CI 72.4 – 100.6 seconds). Furthermore, the mobile fraction within these foci was 0.691 (95% CI 0.688 – 0.694), indicating that Gag within these complexes undergoes rapid exchange with cytoplasmic Gag (Fig. 3.7B). Accordingly, our interpretation is that these foci represent regions of the cytoplasm containing a high local concentration of Gag that undergoes rapid exchange with cytoplasmic Gag and has not yet assembled into a virus particle.

By contrast, the second population of foci (n=5) also exhibited two-phase recovery following bleaching with a fast-phase $t_{1/2}$ of 9.1 seconds (95% CI 7.4 – 11.7 seconds), a slow-phase $t_{1/2}$ of 346.5 seconds (95% CI 285.7 – 440.3 seconds), and a mobile fraction of 0.662 (95% CI 0.657 – 0.668) (Fig. 3.7C). These kinetics indicate that at early timepoints after bleaching, Gag within these foci is rapidly exchanging with cytoplasmic Gag, but at later time points there is slower addition of Gag molecules consistent with the addition of Gag onto an
Figure 7. Assembly of MMTV virus particles in living cells. A) Description of the FRAP assay to measure MMTV assembly in living cells. MMTV Gag was internally labeled with mCherry by inserting the fluorophore into the pp21 domain. The Gag.pp21.imCherry construct was expressed in NMuMG-C3H cells, and live cells were imaged by confocal microscopy. Individual foci were chosen for analysis, and the relative fluorescence of each focus was measured. Foci were monitored for 20 seconds (pre-bleach), subjected to photobleaching, and then monitored for fluorescence recovery for 15 minutes. The graphs show the relative fluorescence intensities of the foci indicated by the red and green circles. Analysis of 19 foci revealed populations of foci with rapid (B), gradual (C), and limited (D) fluorescence recovery after photobleaching. The black line on each graph represents the mean fluorescence intensity from at least four foci, and the grey bars indicate standard error of the mean. Red lines show a the single or double exponential association curve fitted to the averaged data using GraphPad Prism. Individual foci are shown in the orange and green boxes, with the relative fluorescence of the focus shown as the orange or green curve on the graph. E) FRAP analysis of Gag.pp21.imCherry within SGs induced by expression of GFP-G3BP1 or GFP-YB1 (F) in NMuMG-C3H cells. For each condition, a representative cell is shown at the initial pre-bleach timepoint and a single SG chosen for analysis is indicated by the box. Enlarged images of the boxed SG before (pre-bleach), during (bleach), and at various timepoints after photobleaching are shown for both the Gag.pp21.imCherry channel (grayscale) and the GFP channel (green). Graphs show the mean fluorescence intensity for at least five SGs (black line) with standard error of the mean (gray bars). The fluorescence intensities of two representative foci are show as orange and green lines. Data were fit with a double or single exponential association equations (red lines) using GraphPad Prism.
A

Gag pp21 imCh MA mCh pp21 p8 CA NC

Pre-Bleach Bleach Post-Bleach

Relative Fluorescence (AU)

Time (s) 0 500 1000

0 0.5 1.0 1.5

B

Pre Bleach 15.17s 60.15s 300.20s 899.56s

Relative Fluorescence (AU)

Time (s) 0 500 1000

0 0.5 1.0

C

Pre Bleach 15.17s 60.15s 300.20s 899.56s

Relative Fluorescence (AU)

Time (s) 0 500 1000

0 0.5 1.0

D

Pre Bleach 15.17s 60.15s 300.20s 899.56s

Relative Fluorescence (AU)

Time (s) 0 500 1000

0 0.5 1.0

E

Gag pp21 imCherry GFP-G3BP1 Overlay

Pre Bleach 14.59s 30.22s 59.39s 119.83s

Relative Fluorescence (AU)

Time (s) 0 50 100

0 0.5 1.0

F

Gag pp21 imCherry GFP-YB1 Overlay

Pre Bleach 14.59s 30.22s 59.39s 119.83s

Relative Fluorescence (AU)

Time (s) 0 50 100

0 0.5 1.0
ordered complex, such as an assembling virus particle. Indeed, the gradual increase in Gag fluorescence over time is consistent with previous reports describing the kinetics of HIV-1, EIAV, and MLV particle assembly at the plasma membrane [370,371,372,373]. On the other hand, the third population of foci (n=11) exhibited limited, one-phase recovery after photobleaching with a $t_{1/2}$ of 13.4 seconds (95% CI 12.5 – 14.5) and a mobile fraction of 0.25 (95% CI 0.249 – 0.252), suggesting that these foci represent assembled MMTV particles with limited addition of new Gag molecules after bleaching (Fig. 3.7D). Although we present MMTV Gag foci as three distinct populations, we believe that these populations represent distinct points along a continuum of MMTV virus particle assembly, where Gag monomers accumulate in distinct cytoplasmic locations, leading to the assembly of Gag into ordered particles which are subsequently transported through the cytoplasm to the plasma membrane.

Next, we determined the kinetics of Gag mobility within SGs induced by the overexpression of GFP-G3BP1 or GFP-YB1 to ascertain whether Gag within these complexes was assembling into virus particles. Co-expression of Gag.pp21.imCh and GFP-G3BP1 in NMuMG.C3H cells resulted in the accumulation of Gag in SGs as expected (Fig. 3.7E). Within these foci (n=5), Gag exhibited rapid and complete two-phase recovery after photobleaching with a fast phase $t_{1/2}$ of 6.6 seconds (95% CI 3.6 – 39.9 seconds), a slow phase $t_{1/2}$ of 35.1 seconds (95% CI 20.0 – 142.6 seconds), and a mobile fraction of 1.10 (95% CI 1.08 – 1.13). Together, these data indicate that Gag traffics rapidly in and out of
G3BP1-containing SGs, and supports our observation that Gag accumulates within these complexes.

We were surprised to find that recovery of Gag within SGs induced by overexpression of GFP-YB1 exhibited remarkably different kinetics (Fig. 3.7F). The average recovery of Gag fluorescence within these granules (n=5) was best fit by a single exponential equation with a $t_{1/2}$ of 3.3 seconds (95% CI 2.7 to 4.5 seconds) and a mobile fraction of 0.32 (95% CI 0.31 to 0.33). Accordingly, these data indicate that recovery of Gag fluorescence within YB1-containing SGs is dictated by a single binding event. Furthermore, the low mobile fraction signifies that Gag within YB1-containing SGs undergoes only limited exchange with Gag in the cytoplasm. Because Gag and YB1 interact through an RNA intermediate, these data suggest that Gag may bind viral RNA within YB1-containing SGs, although this possibility remains to be formally tested.

3.4 Discussion

Once infected, the cell becomes a molecular battlefield where viral factors are at war with cellular defensive strategies aimed at restricting virus replication. Stress granules form in response to some viral infections and can limit viral replication, however many viruses have evolved to manipulate the SG pathway to promote virus replication [331]. Here, we report that MMTV co-opts YB1, a SG-associated protein, and this RNA-dependent interaction of Gag and YB1 is required for efficient virus production.
YB1 is a major component of cellular messenger ribonucleoprotein complexes (mRNPs), comprising 20-30% of the total mRNP protein content, and is present in both translating and non-translating mRNPs [374,375]. The YB1 protein contains three domains: an N-terminal alanine/proline-rich domain (A/P domain) followed by the central cold shock domain (CSD) and the C-terminal domain [376]. The CSD preferentially binds to CG-rich RNA sequences whereas the CTD either binds RNA in a sequence-independent manner or mediates the formation of YB1 homo-oligomers [376,377]. Interestingly, the amount of YB1 bound to a transcript can alter the three-dimensional structure of the transcript. At low YB1:RNA mass ratios (0.5:1), both the CSD and the CTD bind to RNA, which promotes an extended conformation thought to favor translation [376,378]. However, at higher YB1:RNA mass ratios (8:1), the YB1 CTD is displaced from the RNA and forms homo-oligomers, resulting in compaction of the RNA and translational repression [374,376]. Additionally, depletion of YB1 also causes translational repression *in vitro*, and results in the accumulation of 48S pre-initiation complexes on the RNA [378].

Recent work in hepatitis C virus (HCV) has shown that YB1 regulates the transition between HCV RNA replication and RNA encapsidation. During HCV infection YB1 is relocalized to sites of virus replication, and depletion of YB1 decreases HCV RNA replication while increasing the production of infectious HCV particles [368]. These results are remarkably similar to our finding that MMTV Gag colocalizes specifically with YB1, but not TIA1, in cytoplasmic granules (Fig. 1). Moreover, we found that the interaction between Gag and YB1 is RNA-
dependent (Fig. 3.6), but the cytoplasmic granules containing Gag and YB1 do not dissociate following cyclohexamide treatment (Fig. 3.1), suggesting that the RNA contained within these complexes is translationally repressed. These results are consistent with the observation that the MMTV Ψ signal significantly overlaps with the gag coding region [126], which implies that RNA bound by Gag is unavailable for translation.

Our finding that depletion of YB1 leads to a concomitant reduction in MMTV virion production (Fig. 3.4A) indicates that YB1 plays a critical role in MMTV replication and suggests that MMTV has evolved to use YB1 to facilitate its own replication. In vitro, depletion of YB1 results in translational repression and accumulation of 48S pre-initiation complexes on mRNAs [378], however we did not detect a decrease in steady-state Gag following YB1 knockdown. This finding, combined with the fact that virus production efficiency is calculated relative to intracellular Gag expression, suggests that the defect in particle production following YB1 knockdown is not due to decreased Gag translation. In contrast, our kinetic analysis suggests that YB1 recruits Gag into cytoplasmic complexes where Gag binds the viral RNA or oligomerizes through Gag-Gag interactions. Our working model (Fig. 3.8) predicts that YB1, and perhaps other SG-associated proteins such as G3BP1, bind the viral genome and serve as landmarks used by Gag to identify viral RNAs at sites of capsid assembly. Therefore, we hypothesize that depleting YB1 decreases the formation of cytoplasmic complexes that facilitate the formation of viral assembly complexes, which could result in the mistargeting of Gag to an assembly-incompetent
Figure 3.8. Model depicting the role of YB1 in MMTV replication. Low levels of YB1 bind full-length MMTV RNA to promote translation, but high levels of YB1 bound to the viral RNA sequester the RNA in translationally repressed RNPs. Gag competes with YB1 for binding this translationally repressed mRNA, which serves as a source of genomes for packaging.
pathway. However, we cannot rule out the possibility that YB1 depletion also interferes with a later step in virus production, such as the transport of viral capsids to the plasma membrane, although YB1 depletion did not grossly alter the distribution of Gag foci within MMTV-infected cells (data not shown).

Interestingly, we found that although TIA1 overexpression strongly relocalized Gag to SGs (Fig. 3.2) modulating TIA1 expression did not affect MMTV virus production (Fig. 3.4B, C). This result was surprising because TIA1 facilitates replication of West Nile virus by binding the 3’ terminal stem-loop of the viral minus-strand RNA, which also inhibits SG formation [379,380]. That altered expression of YB1, but not TIA1, adversely affects MMTV virus production carries several implications. Primarily, these data indicate that YB1 is a more prominent regulator of MMTV capsid assembly than TIA1. Furthermore, because TIA1 acts as a nucleating protein for SG formation [257], the observation that TIA1 knockdown does not affect virus production supports our assertion that the cytoplasmic Gag-YB1 complexes are not SGs, and are instead RNPs specific to MMTV. However, because our siRNA knockdown was specific to TIA1, and did not affect expression of TIAR (Fig. 3.4), we cannot rule out the possibility that TIAR may be able to replace the function of TIA1.

An additional interesting finding is that MMTV neither promotes nor prevents SG formation in infected cells. Under steady-state conditions, a small population (~15%) of YB1 is associated with Gag in cytoplasmic foci (Fig. 3.1), however we did not detect YB1- or TIA1-containing SGs under these conditions. Stress granules frequently form in response to virus infection and several viruses,
including Simliki Forest virus (Alphaviridae) [308], transmissible gastroenteritis virus (Coronaviridae) [381], respiratory syncytial virus (Paramyxoviridae) [312], and mammalian orthoreoviruses (Orthoreoviridae) [309] hijack this response to shut down cellular protein synthesis and facilitate synthesis of viral proteins. Although we did not test the effect of acute MMTV infection on SG formation, our data indicate that chronic MMTV infection does not induce SG formation. However, MMTV also does not inhibit the formation of SGs, as we observed YB1- and TIA1-containing SGs in MMTV-infected cells exposed to heat shock (Fig. 3.1). HS-induced SG formation is mediated by eIF2α phosphorylation by eIF2αK1/HRI (heme-regulated inhibitor), which also phosphorylates eIF2α in response to oxidative stress, such as arsenite treatment [reviewed in [333]]. Similarly, overexpression of SG proteins induces SG formation through protein kinase R (PKR)-mediated phosphorylation of eIF2α [274]. Accordingly, our data show that MMTV does not inhibit HRI- or PKR-mediated SG formation.

We were surprised to find that MMTV Gag accumulates in SGs induced by overexpression of SG proteins (Figs. 3.2, 3.3). That this phenomenon is consistent even when proteins that do not strongly interact with Gag were overexpressed (i.e. TIA1) suggests that Gag may be targeted to SGs indirectly via an RNA-dependent mechanism. This conclusion is supported by the findings that deletion of the NC domain or replacing NC with an RNA-independent leucine zipper interaction domain abolishes the accumulation of Gag in SGs (Fig. 3.6B). Surprisingly, Gag retains the ability to traffic to SGs in the absence of the viral genome (Fig. 3.5), suggesting that Gag may traffic to SGs by interacting with
cellular RNAs or by binding a signal in the \textit{gag} coding region of the viral RNA.

Similarly, cellular RNA-binding proteins may also be targeted to SGs through an RNA-dependent mechanism. For example, zipcode-binding protein 1 (ZBP1) does not participate in SG formation but is targeted to SGs through its interaction with zipcode-containing mRNAs [268]. Moreover, ZBP1 stabilizes zipcode-containing transcripts during stress. The finding that overexpression of TIA1, which strongly induces Gag accumulation in SGs (Fig. 3.2), does not adversely affect virus production (Fig. 3.4) suggests that similar to ZBP1, Gag may traffic to SGs to protect the viral RNA from degradation.

Given that MMTV does not inhibit the formation of SGs and, in fact, MMTV infection facilitates the formation of SGs in response to YB1 overexpression, the use of Gag to protect viral RNA from a hostile cellular environment may represent one mechanism used by MMTV to circumvent SG-mediated cellular antiviral defenses. Interestingly, this strategy may be common among other retroviruses or retrovirus-like elements. For example, the mammalian long interspersed nuclear element (LINE)-1 retrotransposon forms large cytoplasmic ribonucleoprotein complexes (RNPs) thought to be replication intermediates [323]. These LINE-1 RNPs contain LINE-1 RNA and the retrotransposon proteins Orf1p and Orf2p, as well as multiple SG-associated proteins [323,382]. Additionally, the yeast retrotransposon Ty3 forms “retrosomes” containing the Ty3 Gag3 protein, retrotransposon RNA, and yeast P-body-associated proteins [170,171,383]. Yeast P-bodies share many functions of mammalian SGs, and the interaction between Ty3 Gag3 and P-body proteins is essential for efficient retrotransposition.
When viewed in light of our data, these results suggest that targeting retroviral or retrotransposon RNA-binding proteins to SGs may be a common mechanism used to circumvent cellular defenses and promote viral or retrotransposon replication.
Chapter 4

Mouse Mammary Tumor Virus RNA Recruits Gag to Cytoplasmic Complexes Containing Stress Granule-Associated Proteins

Darrin V. Bann and Leslie J. Parent

Abstract

Our previous studies demonstrated that MMTV interacts with cellular mRNA binding proteins associated with SGs to facilitate viral replication. Moreover, these interactions were RNA-dependent, suggesting that they may be mediated by the viral RNA. However, it is not known whether MMTV RNA is actually present within these complexes. To determine the subcellular localization of MTMV RNA, we developed a method to fluorescently label a subviral RNA (svRNA) construct using the previously described λN-eGFP system. To accurately recapitulate the trafficking pathway of authentic MMTV RNA, our svRNA contains the Ψ packaging signal (R, U5, and 746 nucleotides of the gag coding region) and the Rem response element (RemRE), which is bound by MMTV Rem to export unspliced viral RNA from the nucleus. We found that in the presence of Rem the svRNA construct was efficiently exported from the nucleus and localized to cytoplasmic complexes containing Gag and SG-associated proteins including YB1. Strikingly, Rem was also present in these complexes, suggesting that Rem, and perhaps other Rev-like proteins, may play a role in the cytoplasmic trafficking of retroviral RNAs.
4.1 Introduction

The retroviral Gag polyprotein orchestrates the assembly of nascent virus particles within an infected cell. All retroviruses share the same first step of particle assembly, where the \( \Psi \) packaging signal on the viral genomic RNA is bound by a dimer of Gag. In the absence of \( \Psi \), Gag will efficiently encapsidate cellular mRNAs in proportion to the abundance of mRNA in the cell [384]. Amazingly, during viral infection Gag encapsidates viral RNA in vast excess to cellular transcripts, despite observations that virus-derived RNA comprises only about 1% of the total RNA in a retrovirus-infected cell [113,385,386,387]. However, the mechanism by which Gag locates its genomic RNA amidst a vast excess of cellular transcripts remains unclear.

Different approaches have been used to determine the precise subcellular location of the initial Gag:RNA interaction. In our laboratory, genetic experiments have revealed a link between nuclear trafficking of RSV Gag and gRNA packaging, suggesting that RSV Gag binds its gRNA in the nucleus [95]. By contrast, imaging studies have revealed colocalization between HIV-1 Gag and its gRNA has been observed in a pericentrisomal location and on the cytoplasmic face of the nuclear membrane [155,156]. Similarly, FIV Gag and its RNA accumulate on the cytoplasmic side of the nuclear envelope, while MLV Gag and its RNA predominantly accumulate on the cytoplasmic face of endosomal vesicles [158]. However, for all of these viruses the initial interaction between Gag and the gRNA occurs within the nucleus or cytoplasm, while assembly of virus particles occurs at the plasma membrane. Because only a few Gag molecules may be
associated with an RNA in the cytoplasm, it may therefore be difficult to detect Gag:RNA complexes at the initial site of the interaction [388].

In contrast to viruses like HIV-1 and RSV, which follow the C-type assembly pathway, mouse mammary tumor virus (MMTV) follows the B/D-type morphogenic pathway, and assembles complete, immature capsids in the cytoplasm, which are subsequently transported to the plasma membrane for budding. As a result, the initial Gag:RNA interaction may occur in the same subcellular location as capsid assembly, making MMTV an excellent model in which to study the early events of retroviral assembly in vivo. Our previous results have demonstrated that assembly of MMTV capsids occurs in conjunction with the stress granule (SG)-associated protein, YB1, and that Gag accumulates in SGs during cellular stress. Strikingly, we also found that the interaction between Gag and YB1 was sensitive to RNase treatment, suggesting that viral RNA may bridge the Gag-YB1 interaction.

Like all retroviruses, MMTV uses full-length, unspliced viral RNA for two roles in the virus replication cycle: as an mRNA to direct the synthesis of the Gag, Gag-Pro, and Gag-Pro-Pol proteins, and as a genomic RNA (gRNA), which is encapsidated into virus particles. MMTV is a complex retrovirus and encodes a trans-acting HIV-1 Rev-like protein called Rem, which exports unspliced viral RNA from the nucleus [19,20]. Rem is translated from a doubly-spliced MMTV RNA and consists of the Env signal peptide (SP) domain, the first 98 amino acids of the Env SU domain (ΔSU), and the C-terminal 41 amino acids of the Env TM domain (ΔTM) [19,20]. All of the Rem functional motifs, including a nuclear localization
signal (NLS), a nucleolar localization signal (NoLS), an arginine-rich motif (ARM) RNA-binding domain, and a nuclear export signal (NES) are contained within SP [19]. Furthermore, evidence suggests that SP must be proteolytically cleaved from the ΔSUΔTM domain for Rem to function [83,85]. Following SP cleavage, the peptide is retrotranslocated from its site of synthesis on the ER into the nucleus where it binds the Rem response element (RemRE) on unspliced MMTV RNA and exports the transcript through a Crm1-dependent mechanism [85,87,88].

Interestingly, several lines of evidence indicate that for HIV-1, Rev may have additional functions beyond RNA export from the nucleus. For example, several reports indicate that Rev promotes translation of unspliced and sub-optimally spliced viral RNAs through a mechanism that is independent of the Rev response element [389,390,391]. Furthermore, Rev has also been implicated in encapsidation of viral gRNA, suggesting that Rev may play an important role in determining the fate of full-length viral RNAs [151,392]. However, the cytoplasmic trafficking of HIV-1 Rev with viral RNA has not been examined. Because Rem bears striking functional homology to Rev, these data suggest Rem may also play an important role in the efficient use of MMTV RNA for translation or encapsidation.

Viral RNA plays an important structural role in the assembly of all retroviral capsids [180]. However, reports from the literature suggest that MMTV may fail to assemble virus particles in the absence of viral RNA containing the Ψ packaging signal [125]. Furthermore, we have been unable to obtain virus-like particles from cells transfected with MMTV Gag-GFP (unpublished data), suggesting that viral
RNA may be required for the efficient assembly of MMTV virus particles. As a result, we sought to determine the trafficking pathways followed by MMTV RNA during virus infection.

4.2 Materials and Methods

4.2.1 Cell culture and transfection

Uninfected normal murine mammary gland (NMuMG) and MMTV-infected NMuMG-C3H cells were cultured in DMEM supplemented with 10% fetal bovine serum as previously described [342] and were transfected using Lipofectamine 2000 according to the manufacturer’s instructions. The myc-PABPc1 and myc-HuR constructs were kind gifts from Michael Malim (King’s College London, UK).

4.2.2 Cloning of the svRNA, Rem-mCherry, and Gag-Pro plasmids

The CMV-R-U5-Δgag-RemRE-4BoxB construct was based on our previously described MMTV Gag-GFP construct [342]. The R-U5 region of the MMTV 5’ UTR was PCR amplified from NMuMG.C3H total cellular DNA using the following primers: 5’ ATC GAT AGA TCT GCA ACA GTC CTA ACA TTC ACC and 5’ ATC GAT GAT ATC TAC CTC TTC TCC GTA GGC G. The PCR-amplified fragment was inserted into BglIII and EcoRV sites in Gag-GFP to create 5’ UTR Gag-GFP intermediate #1. The EcoRV cloning site between the 5’ UTR and the Gag initiation codon was eliminated by QuickChange mutagenesis using primers 5’ CCG CCT ACG GAG AAG AGG TAG GTT ACG GTG A and 5’ TCA CCG TAA CCT ACC TCT TCT CCG TAG GCG to create 5’ UTR Gag-GFP intermediate #2.
The MMTV RemRE was PCR amplified from NMuMG.C3H total cellular DNA using primers 5' ATC GAT GCG GCC GCA GAT CTT AAC GTG CTT TTA AAA and 5' ATC GAT GCG GCC GCG GAT CCA GTA CTA AAA GAT AAT GAT TCA TTT C and inserted into the NotI site in 5' UTR Gag-GFP intermediate #2 to create CMV-R-U5-Gag-GFP-RemRE. This construct was digested with BsrGI and EcoNI, blunted, and religated to create CMV-R-U5-Δgag-RemRE.

To insert BoxB loops into CMV-R-U5-Δgag-RemRE, both this construct and Srprb-mRFP-4BoxB were first transformed into GM272 dam-3 (-) / dcm-6 (-) E. coli (a gift from David Spector, Penn State College of Medicine) [393]. Digesting Srprb-mRFP-4BoxB (a gift from Jan Ellenberg, EMBL) with BsrGI, blunting using a Klenow reaction, and digesting with XbaI isolated the 4BoxB loops. The isolated BoxB loops were inserted into CMV-R-U5-Δgag-RemRE digested with ScaI and XbaI to create CMV-R-U5-Δgag-RemRE-4BoxB.

To create Rem-mCherry, MMTV Rem was PCR-amplified from Rem-GFP (a gift from Jaquelin Dudley, University of Texas at Austin) [19]. The resulting PCR product was restriction digested and inserted into SalI and HindIII sites of mCherry.N2 [342].

4.2.3 Fixation and immunofluorescence

Approximately 18 hours after transfection, cells were fixed in 3.7% paraformaldehyde, permeabilized in 0.25% Triton-X100, and blocked in 10% bovine serum albumen as described previously [342]. After blocking, cells were incubated with one or more of the following primary antibodies: mouse anti-MMTV
CA (a gift from Tanya Golovkina, University of Chicago), rabbit anti-YB1 (AbCam), rabbit anti-TIA1 (AbCam), rabbit anti-myc (AbCam), or rabbit anti-G3BP1 (Bethyl Laboratories). After washing, samples were then incubated with appropriate anti-mouse-Cy3 or –Cy5 or anti-rabbit-Cy5 conjugated secondary antibodies (Invitrogen). Nuclei were visualized by staining with 4’6-diamidino-2-phenylindole (DAPI). Samples were imaged using Leica SP2 or SP8 ABOS laser scanning confocal microscopes. Images were processed for publication using Corel Draw (Corel Corp. Ottawa, CA), and any adjustments to image intensity were made equally to all images in a series. Colocalization colormaps were generated using the Colocalization Colormap plugin for Image J [359,361].

4.3 Results

4.3.1 MMTV RNA and Gag colocalize in cytoplasmic granules

To visualize the trafficking pathways of MMTV RNA, we inserted four BoxB stem-loops into a subviral RNA (svRNA) construct driven by the CMV promoter and containing R, U5, the first 746 nucleotides of the gag coding region, and the Rem response element (RemRE) (Fig. 4.1A) [394,395]. The RNA can be visualized by co-expressing a fusion protein consisting of four copies of the bacteriophage $\lambda_N$ coat protein fused to three copies of eGFP ($\lambda_N$-eGFP), which tightly and specifically binds the BoxB stem-loops in the RNA [394,395,396]. Importantly the $\lambda_N$-eGFP reporter contains a H3 nuclear localization signal and therefore when the reporter is expressed alone in MMTV-infected NMuMG-C3H cells, it is confined to the nucleus (Fig. 4.1B). Strikingly, when the svRNA was
Figure 4.1 Localization of MMTV Gag and RNA to cytoplasmic granules. (A) Cartoon depicting the svRNA bound by the $\lambda_N$-eGFP reporter. (B) $\lambda_N$-eGFP expressed without RNA in NMuMG-C3H cells. (C) Two optical slices of the same NMuMG-C3H cell expressing the svRNA, $\lambda_N$-eGFP, and immunostained for Gag using an $\alpha$-CA antibody. Gag and the svRNA colocalize in small complexes (top row) and larger granules (bottom row). Enlargements of the area indicated by the box are shown to the right. (D) A non-viral control RNA (Srprb-mRFP-4BoxB) [395] expressed in NMuMG-C3H cells immunostained for Gag using $\alpha$-CA.
co-expressed with $\lambda^N$-eGFP in MMTV-infected cells, which provide all of the trans-acting factors required for RNA trafficking, the reporter was exported from the nucleus and accumulated in cytoplasmic granules (Fig. 4.1C). Interestingly, Gag also accumulated in these granules, suggesting that these complexes may represent sites of MMTV capsid assembly (Fig. 4.1C). Importantly, the distribution of Gag was unaffected by the expression of a non-viral RNA, Srprb-mRFP-4BoxB, indicating that the accumulation of Gag and RNA in cytoplasmic granules is specific to the svRNA construct (Fig. 4.1D).

4.3.2 Stress granule-associated proteins are present in MMTV RNA granules

Our previous results indicated that MMTV Gag and YB1 interact through an RNA intermediate (Chapter 3). Therefore, to test whether YB1 was also present in cytoplasmic granules with Gag and svRNA, we expressed $\lambda^N$-eGFP and the svRNA in MMTV-infected NMuMG-C3H cells and immunostained for Gag and YB1. Strikingly, expression of the svRNA caused YB1 to accumulate in cytoplasmic granules with Gag and the viral RNA, suggesting that viral RNA does indeed mediate the interaction between Gag and YB1 (Fig. 4.2A). To test whether this interaction was specific to MMTV viral RNA we expressed a non-viral RNA, Srprb-mRFP-4BoxB, in MMTV-infected NMuMG-C3H cells [395]. Expression of the Srprb-mRFP-4BoxB RNA is confirmed by production of Srprb-mRFP fluorescent protein, which localizes to the ER. Importantly, Srprb-mRFP-4BoxB did not alter the distribution of endogenous YB1 or Gag, indicating that the accumulation of Gag and YB1 in granules is specific to the svRNA (Fig. 4.2B)
Figure 4.2 MMTV RNA granules contain SG-associated proteins. (A) MMTV svRNA and λN-eGFP expressed in NMuMG-C3H cells immunostained for Gag and endogenous YB1 (top), expressing myc-PABPc1 (middle), or expressing myc-HuR (bottom) and immunostained using an anti-myc specific antibody. (B) MMTV-infected NMuMG-C3H cells expressing a non-viral RNA construct, Srprb-mRFP-4BoxB [395] and immunostained for endogenous YB1 and Gag. Expression of the non-viral RNA is marked by the production of Srprb-mRFP protein, which localizes to the endoplasmic reticulum.
To test whether other SG-associated proteins were also present within these complexes, we coexpressed $\lambda_N$-eGFP, the svRNA, and myc-PABPc1 or myc-HuR in NMuMG-C3H cells. Interestingly, both myc-PABPc1 and myc-HuR accumulated in complexes containing Gag and the svRNA, suggesting other SG-associated proteins may also localize to these complexes. Together, these data indicate that MMTV RNA may bind the viral RNA at sites of capsid assembly.

### 4.3.3 Gag-independent trafficking of RNA to cytoplasmic granules

To better understand the mechanisms underlying the cytoplasmic trafficking of MMTV RNA, we next tested whether Gag influenced MMTV RNA trafficking by expressing the svRNA in uninfected NMuMG cells. As expected, when $\lambda_N$-eGFP and the svRNA were co-expressed without Rem, the reporter was not exported from the nucleus (Fig. 4.3A). Moreover, the distribution of YB1 in these cells was not significantly altered, indicating that the formation of YB1 granules is dependent on the accumulation of svRNA in the cytoplasm (Fig. 4.3A).

When the svRNA and reporter were co-expressed with MMTV Rem-mCherry, however, the RNA was readily exported from the nucleus and accumulated in cytoplasmic granules with YB1, indicating that the RNA and Rem are sufficient to accumulate in cytoplasmic granules in the absence of Gag (Fig. 4.3B). Interestingly, we also observed Rem-mCherry in these granules (Fig. 4.3B). This result was surprising because previous reports have indicated that the N-terminal 98 amino acids of Rem comprising the signal peptide (SP) are responsible for nuclear export of unspliced MMTV RNA and that SP must be
Figure 4.3 MMTV Rem localizes to cytoplasmic granules with the svRNA. (A) A cartoon depicting the Rem-mCherry construct. Amino acid numbers are indicated above the protein. The signal peptide (SP) domain, containing the NLS, NoLS, NES, and ARM [19] is shaded grey. (B) Expression of the svRNA and $\lambda_N$-eGFP without Rem in uninfected NMuMG cells immunostained for YB1. (C) An uninfected NMuMG cell expressing svRNA, $\lambda_N$-eGFP, and Rem-mCherry and immunostained for YB1. The top row shows the distribution of Rem-mCherry, while the bottom row shows the distribution of YB1 within the same cell. Colocalization colormaps were generated using the Colocalization Colormap [359] plugin for Image J [361] and are shown as “colocalization.”
cleaved from the remainder of Rem to export RNA from the nucleus [83]. Because our Rem-mCherry construct contains a fluorophore on the C-terminus of the protein, our result suggests that full-length Rem may play a role in the nuclear export and cytoplasmic trafficking of MMTV RNA.

4.3.4 MMTV RNA recruits Gag to cytoplasmic granules containing YB1

To determine the effect of viral RNA on Gag localization, we first expressed a Rem-independent Gag-Pro construct in uninfected NMuMG cells. This construct contains the ribosomal frameshift site present within exogenous C3H MMTV, and should therefore produce Gag in the expected 3:1 ratio (Fig. 4.4A) [107,219]. Interestingly, Gag-Pol formed small foci in the cytoplasm, however these foci only partially colocalized with YB1 (Fig. 4.4B), similar to our previous observations using Gag-GFP (Chapter 3). However, when Gag-Pol was co-expressed with svRNA and Rem, the viral RNA, Gag, and YB1 colocalized in large complexes near the nucleus and smaller complexes near the periphery of the cell (Fig. 4.4C). As a whole, these data indicate that the viral RNA recruits Gag to sites of RNA accumulation, which may serve as sites of gRNA encapsidation.
Figure 4.4 MMTV RNA recruits Gag to cytoplasmic granules. (A) A cartoon depicting the MMTV Gag-Pro 4xCTE construct. The frameshift between Gag and Pro is indicated by the broken line. (B) An uninfected NMuMG cell transfected with MMTV Gag-Pro and immunostained for Gag (α-CA) and endogenous YB1. (B) An uninfected NMuMG cell transfected with MMTV Gag-Pro, svRNA, λN-eGFP, and untagged Rem. The top row shows the distribution of Gag while the bottom row shows the distribution of YB1. Colocalization colormaps were generated as above and are shown as “colocalization”.
4.4 Discussion

The encapsidation of genomic RNA is essential for retroviral replication, however the intracellular landmarks that retroviruses use to efficiently locate their own genomes amidst an excess of cellular transcripts is poorly understood. Our data indicate that MMTV RNA and Gag colocalize in large cytoplasmic granules and in small cytoplasmic foci (Fig. 4.1). Strikingly, bright foci of MMTV Gag are frequently localized within or adjacent to the large granules, suggesting that these may represent sites of capsid assembly, while the small cytoplasmic foci represent immature virus particles containing labeled svRNA. Interestingly, SG-associated proteins also colocalize with the RNA and Gag within the large complexes, suggesting that SG-associated proteins may play a role in the selection of MMTV RNA for encapsidation (Fig. 4.1).

The other notable observation regarding this finding is that the formation of large RNA granules is specific to expression of the svRNA, as a non-viral RNA, srprb-mRFP, did not accumulate in similar complexes. We have previously noted that MMTV infection primes cells to form SGs in response to YB1 overexpression (Chapter 3), however the svRNA promotes the formation of RNA granules in both infected and uninfected NMuMG cells, suggesting that an intrinsic property of this RNA promotes granule formation. The cis-acting sequences responsible for targeting transcripts to SGs are not well understood [269], so this MMTV RNA may provide an important tool for studying how cellular transcripts are targeted to SGs during stress.
One mechanism by which cellular transcripts can be targeted to SGs is through AU-rich elements (AREs) typically located in the 3’ untranslated region of the transcript. These sequences, consisting of dispersed or tandem AUUUA pentamers, are bound by a number of SG-associated proteins including YB1, HuR, and tristetraprolin (TTP), which control the stability and translational state of the transcript [397,398,399,400]. Interestingly, the MMTV svRNA contains two AUUUA pentamers and both YB1 and HuR are among the proteins that colocalize with the svRNA (Fig. 4.2), suggesting that ARE-binding proteins may help target the viral RNA to SG-like complexes. Furthermore, YB1 and HuR are both implicated in stabilizing transcripts to which they are bound [401], suggesting that MMTV may have evolved to use these factors to help protect the viral RNA from degradation.

Interestingly, the presence of Rem within complexes containing viral RNA (Fig. 4.3) also suggests that Rem may play a role in the cytoplasmic trafficking of viral RNA. Although functions of Rem beyond nuclear export of unspliced MMTV RNA have not been reported, evidence in HIV-1 indicates that Rev is involved in targeting viral transcripts for translation and/or encapsidation [151,391,392]. However, the subcellular trafficking of Rev bound to viral RNA has not been examined. As a result, our data provide the first imaging-based evidence that a retroviral Rev-like protein remains associated with the viral transcript in the cytoplasm.

We were surprised to find that expression of the MMTV RNA also influenced the trafficking of Gag. The cis- and trans-acting factors that control
MMTV Gag localization are not well characterized. However, the Gag protein MPMV, which assembles in the cytoplasm similarly to MMTV, contains a cytoplasmic targeting/retention signal (CTRS), which targets Gag to pericentrosomal sites for capsid assembly [168,169]. Interestingly, MMTV contains a putative CTRS that is able to functionally substitute for the MPMV CTRS, however CTRS activity in the context of MMTV Gag has not been demonstrated. Our data demonstrate that Gag is capable of forming small complexes in association with YB1, however co-expression of a Ψ'-containing RNA resulted in a dramatic redistribution of Gag to large complexes containing the viral RNA (Fig. 4.4). Together, these data suggest that MMTV RNA may be a primary determinant of intracellular Gag trafficking, at least at the early stages of immature virus particle assembly. As a result, we propose a model of MMTV assembly where translationally-repressed Ψ'-containing viral RNAs recruit Gag to cytoplasmic ribonucleoprotein complexes, which serve as sites of immature particle assembly.
Chapter 5
Proteins Associated with P-bodies and RNA-Induced Silencing Complexes Facilitate MMTV Assembly

Darrin V. Bann, Andrea R. Beyer, and Leslie J. Parent

Note:
Figure 5.5 was previously published in

Abstract
The data presented in chapters 3 and 4 indicate that cytoplasmic assembly of MMTV capsids occurs in conjunction with specific mRNA binding proteins associated with SGs. However, eukaryotic cells also possess other types of messenger RNPs, including P-bodies and RISCs, which are generally associated with mRNA decay. However, data from the yeast Ty3 retrotransposon, which follows an assembly pathway similar to MMTV, must interact with P-body proteins to replicate efficiently. Furthermore, many proteins associated with SGs may also localize to P-bodies and/or RISCs. Together, these observations led us to test whether proteins associated with P-bodies also interact with the MMTV assembly pathway. Interestingly we found that overexpression of the P-body associated protein Mov10, and the RNA interference effector molecules Ago1 and Ago2, caused MMTV Gag to accumulate in large P-bodies/RISCs. By contrast, Gag did not accumulate in P-bodies when Dcp1a, a core P-body protein, was overexpressed. Interestingly, however, a subviral MMTV RNA construct transiently colocalized with Dcp1a, suggesting that viral RNA may transiently localize to Dcp1a-containing P-bodies. Depletion of Mov10 using siRNAs reduced MMTV virus production, indicating that Mov10 is required for efficient MMTV replication. However, overexpression of Mov10 did not affect virus production, indicating that Mov10 is not a restriction factor for MMTV. Interestingly, Gag proteins from diverse mammalian retroviruses also colocalized with Mov10, consistent with reports that Mov10 plays a role in the replication of several exogenous retroviruses.
5.1 Introduction

All retroviruses use full-length, unspliced viral RNA (vRNA) for two roles in the viral replication pathway: as an mRNA to direct the synthesis of the Gag, Gag-Pro, and Gag-Pro-Pol proteins; and as a genomic RNA (gRNA), which is encapsidated into nascent virus particles. Selection of viral RNA is mediated by a high affinity interaction between the Ψ encapsidation signal on the vRNA, and the NC domain of Gag [402]. Accordingly, viral RNA is encapsidated in vast excess to cellular transcripts, despite the observation that viral RNAs comprise only about 1% of the poly-adenylated mRNA within the cell [113,386,387]. These observations suggest that a mechanism may exist for retroviral Gag proteins to efficiently locate and identify viral transcripts for packaging, however the intracellular landmarks used by Gag to recognize potential genomes remain unclear.

Post-transcriptional control plays a critical role in the regulation of eukaryotic gene expression. The effector molecules of post-transcriptional regulation are cellular RNA binding proteins that form ribonucleoprotein complexes (RNPs) including processing bodies (P-bodies), RNA-induced silencing complexes (RISCs), and stress granules (SGs). In general, P-bodies are characterized by the presence of the Dcp1a/Dcp2 mRNA decapping complex and the Xrn1 5' → 3' exonuclease, and therefore are thought to represent sites of mRNA decay [292,293,294,295,403]. On the other hand, RISCs are involved in translational regulation of specific transcripts targeted by micro-RNAs (miRNAs) [404]. Accordingly, RISCs are characterized by argonaute (Ago)-family proteins,
the effector molecules of RNA interference, and GW182, which is required for RISC function, but lack core P-body proteins including Dcp1 [306,405,406,407]. It is important to note, however, that RISC proteins including Ago family members and GW182 are also present within P-bodies, suggesting that P-bodies and RISCs represent different points along continuum of RNPs [408].

Interestingly, proteins associated with P-bodies and RISCs may play important roles in retroviral replication. Notably, the retrovirus-like yeast retrotransposons Ty1 and Ty3 assemble cytoplasmic capsids in RNPs called “retrosomes”, which contain multiple P-body proteins [170,171,321,322]. Among metazoan retroviruses, modulating expression of the P-body protein Mov10 adversely affects HIV-1 virus production and infectivity [327,328,329]. Additionally, HIV-1 co-opts the P-body associated RNA helicase Ddx6 (Rck/p54) to facilitate capsid assembly, and HIV-1 and PFV Gag proteins recruit Ago2 to viral RNAs through an miRNA-independent mechanism [319,320]. As a whole, these findings suggest that diverse retroviruses hijack many components of P-bodies or RISCs to facilitate viral replication, however the precise mechanism by which many of these proteins facilitate replication remains unknown.

Mouse mammary tumor virus (MMTV) is an oncogenic retrovirus that assembles complete, immature capsids in the cytoplasm of infected cells. However, the cellular proteins associated with sites of MMTV capsid assembly are not well characterized. Given the role of P-body proteins in metazoan retrovirus replication, and the observation that the MMTV replication pathway is phenotypically similar to that of Ty3, we investigated whether MMTV interacts with
P-body proteins to facilitate its own replication. Strikingly, we found that MMTV interacts with several P-body proteins including Mov10 and Ago2. However, the MMTV assembly complexes excluded Ddx6, suggesting that MMTV assembly does not occur in P-bodies per se.

5.2 Materials and Methods

5.2.1 Cell culture and transfection

Normal murine mammary epithelial (NMuMG) (ATCC CRL-1636) and MMTV-infected NMuMG (NMuMG-C3H) [352,353] cells were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone). HeLa cells were cultured in DMEM with 5% FBS and supplemented with sodium pyruvate and sodium bicarbonate (HyClone), and QT6 cells were cultured as described previously [354,355]. NMuMG and HeLa cells were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions, and QT6 cells were transfected using the calcium phosphate method [355].

The myc-Mov10 plasmid was a gift from Michael Malim (Kings College London, UK). Myc-Ago1 and myc-Ago2 were gifts from Greg Hannon (Cold Spring Harbor Laboratory, NY), and RFP-Dcp1a was a gift from Paul Anderson (Harvard Medical School, MA). The MMTV Gag-GFP, GagΔNC-GFP, and svRNA constructs were described previously (Chapter 2; Chapter 4). The λN-eGFP plasmid was a kind gift from Jan Ellenberg (EMBL) [395].
5.2.2 Small interfering RNA (siRNA) knockdown and virus production assays

Small interfering RNA knockdowns were performed using siRNAs targeting murine Mov10 (Invitrogen) or scrambled control siRNAs (Sigma). The siRNA transfections and virus release assays were conducted as described previously (Chapter 2). Knockdown of Mov10 was confirmed by Western blotting using an anti-Mov10 primary antibody (AbCam) plus an anti-rabbit HRP-conjugated secondary antibody (Sigma). An anti-GAPDH antibody (GenScript) was used as a loading control. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and imaged using an EC3 Chemi HR 410 imaging system (UVP, Upland, CA). Virus production assays were conducted a minimum of four times for each condition, and the mean, standard error of the mean, and p-values were calculated using GraphPad Prism (GraphPad Software, Inc.).

5.2.3 Microscopy

Cells were fixed in 3.7% paraformaldehyde 15 to 18 hours after transfection. Samples were permeabilized using 0.25% TritonX-100, and blocked in 10% bovine serum albumen. Immunostaining was conducted using mouse anti-MMTV CA [409], rabbit anti-myc (AbCam), rabbit anti-Mov10 (AbCam), rabbit anti-Ddx6 (AbCam), or human anti-18033 (a kind gift from Marvin Fritzler, University of Calgary) with appropriate anti-mouse Cy3 (Invitrogen), anti-rabbit
Cy5 (Invitrogen), or anti-human Cy5 (AbCam) secondary antibodies. Samples were imaged using a Leica SP2 ABOS Laser Scanning Confocal Microscope.

Live cell imaging was conducted using a DeltaVision DV Elite deconvolution microscope fitted with a 60x 1.4 NA objective in a 37°C environmental chamber (Applied Precision, WA). Cells were cultured in phenol red-free DMEM (HyClone) supplemented with 10% fetal bovine serum and 25 mM HEPES. A series of 6 Z-positions were imaged through the center of each cell with a step size of 0.2 µm and cells were imaged every minute for 20 minutes. Images were deconvolved using softWoRx Suite 2.0 (Applied Precision, WA) and the middle 4 slices were used to make a maximum projection.

Images were processed for publication using Corel Draw (Corel Corp. Ottawa, CA), and any adjustments to image intensity were made equally to all images in a series. Colocalization colormaps were generated using the Colocalization Colormap plugin for Image J [359,361].

5.2.4 Co-immunoprecipitations

Co-immunoprecipitations were conducted essentially as described (Chapter 2; Chapter 3). Protein A sepharose beads (Invitrogen) were prepared by washing twice with Co-IP buffer (50 mM Tris pH 8.0, 1% Triton-X100, 0.1% SDS, 1% deoxycholic acid, 150 mM NaCl) and incubating with rabbit α-Mov10 (AbCam) or rabbit anti-Lamin A/C (Santa Cruz Biotechnology). Cells were lysed in lysis buffer (50 mm Tris pH 7.05, 1% Triton-X100, 0.5% deoxycholic acid, 150 mM NaCl) and prior to conducting the Co-IP, some samples were treated with 25 µg
RNaseA (Invotrogen) for 15 minutes, while other samples were left untreated and added to prepared beads. Samples were incubated overnight at 4°C with gentle end-over-end mixing. Beads were washed twice with Co-IP buffer and twice with TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0). Protease inhibitor cocktail (Sigma) and PMSF (G-Biosciences) were added to all buffers.

Proteins were eluted from beads by boiling in sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 0.5% bromophenol blue, 4% SDS, and 10% β-mercaptoethanol), separated by SDS-PAGE, and transferred to PVDF membranes (BIORAD). Western blotting was performed using mouse α-CA antibody followed by goat α-mouse-horseradish peroxidase conjugate antibody (Sigma). Blots were developed using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific).

5.3 Results

5.3.1 MMTV Gag colocalizes with endogenous P-body components

Assembling MMTV capsids form discrete cytoplasmic foci. To examine whether P-body or RISC proteins were associated with these sites of assembly, we immunostained MMTV-infected cells for Gag and endogenous P-body and/or RISC proteins. Interestingly, staining revealed that endogenous Mov10 did partially colocalize with some Gag foci, suggesting that Mov10 may be present at sites of MMTV capsid assembly (Fig. 5.1). Similarly, when cells were stained with the human serum 18033, which contains antibodies to GW182 and the core
**Figure 5.1 Colocalization of MMTV Gag with P-body components.** MMTV-infected NMuMG cells were immunostained for Gag and four different P-body markers including Mov10 (top), Ge-1 and GW182 using antibody 18033 (middle), or Ddx6 (bottom). Enlarged images of the areas indicated by boxes are shown to the right.
P-body protein Ge-1 [410], Gag also partially colocalized with cytoplasmic foci stained with the 18033 serum. However, because antibodies in this serum recognize antigens present in both P-bodies and RISCs, this result did not allow us to conclude whether MMTV Gag foci were associated with P-bodies or RISCs specifically. To more directly assess whether P-bodies or RISCs were associated with sites of MMTV assembly, we immunostained MMTV-infected cells with a Ddx6-specific antibody. As expected, the α-Ddx6 antibody stained small cytoplasmic foci, which have previously been shown to represent P-bodies [270,281]. However, while Gag did not strongly colocalize with Ddx6, foci of Gag were frequently adjacent to Ddx6 foci, which is reminiscent of “docking” observed between P-bodies and SGs [274].

5.3.2 Overexpression of P-body proteins causes MMTV Gag to accumulate in P-bodies

We observed previously that inducing SG formation by overexpressing SG-associated proteins caused MMTV Gag to accumulate in large SGs (Chapter 3). To test whether overexpression of P-body proteins would produce a similar phenotype, we overexpressed P-body proteins in NMuMG-C3H cells, which were then fixed and immunostained for Gag. Interestingly, overexpression of Mov10 resulted in the formation of large cytoplasmic granules containing myc-Mov10 (Fig. 5.2A). Furthermore, in cells overexpressing Mov10, Gag was dramatically re-distributed to Mov10-containing granules. Mander’s analysis revealed that 76% of the myc-Mov10 was associated with Gag (M₁), while 49% of Gag
Figure 5.2 Trafficking of Gag to P-bodies and RISCs. (A) MMTV-infected NMuMG-C3H cells transiently transfected with myc-Mov10 (top) or RFP-Dcp1a (bottom). Gag and myc-Mov10 were visualized by immunostaining with anti-CA and anti-myc specific antibodies. (B) NMuMG-C3H cells expressing the RISC effector molecules myc-Ago1 (top) or myc-Ago2 and immunostained for Gag and myc. Colocalization colormaps were generated using the Colocalization ColorMap plugin [359] for ImageJ [361]. Colormaps are shown under “colocalization” and enlarged images of the areas indicated by squares are shown under “enlargement”
overlapped with Mov10 (M2). Furthermore, colocalization colormapping demonstrated an accumulation of Gag and Mov10 in cytoplasmic granules, indicating that overexpression of Mov10 causes Gag to accumulate in P-bodies (Fig. 5.2A).

By contrast, overexpression of the canonical P-body protein Dcp1a tagged with RFP (RFP-Dcp1a), resulted in the formation of large P-bodies, however Gag did not strongly accumulate in these complexes. In fact, Mander's analysis indicated only 6% overlap between RFP-Dcp1a and Gag, and only 5% overlap between Gag and Dcp1a (Fig. 5.2A). Moreover, colocalization colormapping revealed that Gag was largely excluded from Dcp1a-induced P-bodies. However, Gag did colocalize with Dcp1a around the periphery of P-bodies (Fig. 5.2A, enlargement), similar to the distribution of Gag and endogenous Ddx6 (Fig. 5.1). Similar “docking” interactions are observed between SGs and P-bodies, potentially to exchange RNA between the two complexes [274]. As a result, these data indicate that MMTV Gag may transiently interact with P-bodies per se.

To examine whether MMTV Gag was also associated with RISCs, we overexpressed myc-tagged Ago1 or Ago2. Interestingly, overexpression of Ago1 also induced the accumulation of Gag in discrete complexes containing myc-Ago1. However, within these complexes Gag frequently did not directly overlap Ago1, but rather was juxtaposed to Ago1 (Fig. 5.2B). This observation was confirmed by colocalization colormapping, which showed relatively modest colocalization between Gag and Ago1 within Ago1-containing RISCs (Fig. 5.2B). In contrast to Ago1, myc-Ago2 did not form large complexes when overexpressed,
but rather remained more diffuse in the cytoplasm (Fig. 5.2B). However, myc-Ago2 overlapped more directly with cytoplasmic Gag foci than did myc-Ago2 (Fig. 5.2B, enlargement). Although the reason underlying difference between the colocalization of Gag with Ago1 and Ago2 is not known, these data suggest that Gag may differentially interact with Ago1 or Ago2 during MMTV capsid assembly. Accordingly, we conclude that overexpression of specific P-body or RISC proteins differentially affects Gag localization in MMTV-infected NMuMG cells.

5.3.3 Gag is sufficient to colocalize with P-body proteins

To examine whether Gag was sufficient to traffic to P-bodies or RISCs, we coexpressed MMTV Gag-GFP (Fig. 5.3A) (Chapter 2) and myc-Mov10, myc-Ago1, or myc-Ago2 in uninfected NMuMG cells. Strikingly, Gag-GFP colocalized with all three proteins (Fig. 5.3B), indicating that Gag is sufficient to traffic to P-bodies in the absence of other viral proteins. We found previously that deletion of the Gag NC domain, which is required for both Gag-Gag and Gag-RNA interactions, disrupted the ability of Gag to localize to SGs (Chapter 3). However, when we co-expressed GagΔNC-GFP (Fig. 5.3C) (Chapter 2) with myc-Mov10, myc-Ago1, or myc-Ago2, we were surprised to observe that the Gag truncation mutant retained the ability to traffic to P-bodies and RISCs. These data suggest that Gag trafficking to P-bodies may be mediated through a domain other than NC, or that the interaction between Gag and P-body proteins is RNA-independent.
Figure 5.3 NC-independent localization of Gag to P-bodies and RISCs. (A) Cartoon depicting MMTV Gag-GFP. (B) MMTV Gag-GFP was coexpressed with myc.Mov10, myc-Ago1, or myc-Ago2 in uninfected NMuMG cells immunostained for myc. Colocalization colormaps are shown on the right under “colocalization”. (C) The MMTV GagΔNC-GFP construct. (D) Uninfected NMuMG cells expressing GagΔNC-GFP and myc.Mov10, myc.Ago1, or myc.Ago2. Cells were immunostained for myc using a specific antibody. Colocalization colormaps are shown under “colocalization”. 
5.3.4 RNA-dependent interaction between Gag and Mov10

To directly determine whether RNA is required for the interaction between Gag and Mov10, we conducted coimmunoprecipitations (CoIPs) using an anti-Mov10 specific antibody. We found that Gag coimmunoprecipitated with Mov10, however, we were surprised to find that treating the samples with RNase prior to conducting the CoIP disrupted the interaction between Gag and Mov10 (Fig. 5.4). Accordingly, these data indicate that the Gag-Mov10 complex is RNA dependent, although Gag may not depend upon RNA for localization to P-bodies.

5.3.5 MMTV RNA transiently localizes to P-bodies

To this point, our data indicated that MMTV Gag traffics to P-bodies through an RNA-independent mechanism, but that RNA is required for Gag to interact with the P-body protein Mov10. Because Gag binds the Ψ sequence in viral RNA with high affinity and specificity, these results suggest that MMTV RNA may localize to P-bodies and/or RISCs. To directly visualize the trafficking of MMTV RNA with respect to P-bodies, we utilized a previously described subviral RNA (svRNA), which is bound by the λN-eGFP reporter (Fig. 5.5A) (Chapter 4; [395]). As expected, when λN-eGFP was expressed alone in uninfected NMuMG cells, the reporter localized to the nucleus (Fig. 5.5B). Similarly, when the reporter and RNA were expressed without Rem, λN-eGFP was not exported from the nucleus. However, co-expression of the svRNA, Rem, and λN-eGFP resulted in the reporter being exported from the nucleus, and the accumulation of RNA in cytoplasmic foci (Fig. 5.5B, arrow).
Figure 5.4 RNA-dependent interaction of Gag with Mov10. A Mov10-specific antibody was used to immunoprecipitate Mov10 from lysates of MMTV-infected NMuMG-C3H cells and an anti-Lamin A/C antibody served as an isotype control (IgG). Prior to conducting the immunoprecipitation, some samples were treated with 25 µg RNase A for 15 minutes at room temperature, or were left untreated. Input (2%), unbound (2%), and antibody-bound fractions were probed for Gag (arrow) by Western blotting using an anti-CA antibody.
Figure 5.5 MMTV RNA transiently colocalizes with RFP-Dcp1a. (A) A cartoon depicting the MMTV svRNA construct containing R, U5, and 746 nucleotides of the gag coding sequence (Ψ), the Rem response element (RemRE), and four BoxB loops, which are bound by the λN-eGFP reporter. (B) NMuMG cells transfected with λN-eGFP; λN-eGFP and svRNA; or λN-eGFP, svRNA, and Rem. The arrow points to svRNA granules in the cytoplasm. (C) Still images from a live-cell timelapse showing transient colocalization of svRNA and RFP-Dcp1a. The time of each image during the timelapse is shown in the upper left-hand corner. Separate green and red arrowheads indicate svRNA and RFP-Dcp1a, respectively, while yellow arrowheads point to colocalized svRNA and RFP-Dcp1a. Figure reproduced from [394].
To visualize the dynamic trafficking of MMTV RNA and P-bodies, we co-expressed \( \lambda_N \)-eGFP, the svRNA, Rem, and RFP-Dcp1a in NMuMG cells. We then imaged a 6-slice Z-stack every minute for 20 minutes to track the viral RNA and P-bodies. Interestingly, after 5 minutes we observed a small focus of RNA just outside the nucleus (Fig. 5.5C). Over the next four minutes, this RNA focus migrated to a nearby P-body labeled with RFP-Dcp1a. The RNA then proceeded to co-traffic with the P-body for four more minutes, before exiting the P-body and resuming an independent trafficking pathway (Fig. 5.5C). As a result, these data indicate that MMTV RNA may transiently traffic to P-bodies in the absence of Gag.

5.3.6 Functional role of Mov10 in MMTV virus production

Previous reports have indicated that modulation of Mov10 expression adversely affects HIV-1 virus production and infectivity \([327,328,329]\). To assess whether depletion of Mov10 also affected MMTV virus production, we used Mov10-specific siRNAs to reduce Mov10 expression by 80% relative to cells treated with a scrambled control siRNA (Fig. 5.6A). Strikingly, this reduction in Mov10 expression led to a 55% reduction in MMTV virus production, indicating that Mov10 is an essential cofactor required for efficient virus production. By contrast, overexpressing Mov10 by 7-fold had no effect on virus production (Fig. 5.6B), suggesting that Mov10 does not play a role in inhibiting MMTV replication. As a result, we conclude that MMTV has evolved to hijack Mov10 to facilitate its own replication.
Figure 5.6 Effect of Mov10 expression levels on MMTV virus production. (A) Graphs showing relative Mov10 expression (left) or MMTV virus production (right) in NMuMG-C3H cells treated with Mov10 siRNA or scrambled control siRNA. Mov10 expression was determined relative to GAPDH loading controls, and the average Mov10 expression level from cells treated with scrambled siRNA was set to 100%. Virus production was determined as the signal from Gag released into the media over the total signal of media plus Gag in the lysate (V/V+L), and was set relative to cells treated with scrambled control siRNA. (B) Graphs showing Mov10 expression (left) and relative virus production (right) in NMuMG-C3H cells that were transfected with GFP or myc-Mov10.
5.3.7 Diverse mammalian retroviral Gag proteins interact with Mov10

We found that overexpression of Mov10 relocalizes MMTV Gag to large P-bodies in MMTV-infected cells, and that MMTV Gag-GFP was sufficient to colocalize with Mov10 in the absence of other viral factors. Interestingly, Mov10 has also been implicated in promoting or inhibiting the replication of several other mammalian retroviruses, including HIV-1, Simian immunodeficiency virus (SIV), MLV, and M-PMV [326,327,328,329,411]. However, whether Mov10 overexpression affects Gag localization in these viruses is not known. Accordingly, we tested the effect of Mov10 overexpression on MLV, HIV-1, and RSV Gag localization.

As a control, we first co-expressed MMTV Gag-GFP with myc-Mov10 in uninfected NMuMG cells and, as expected, MMTV Gag-GFP and myc-Mov10 colocalized in cytoplasmic foci (Fig. 5.7). In NMuMG cells expressing MLV Gag-GFP, Gag was predominantly localized to the cytoplasm as expected, but surprisingly myc-Mov10 also colocalized with Gag-GFP at the plasma membrane (Fig. 5.7). Additionally, myc-Mov10 also formed large cytoplasmic granules, which accumulated low levels of MLV Gag-GFP (Fig. 5.7). Similarly, in HeLa cells expressing HIV-1 Gag-GFP, Gag and myc-Mov10 were also colocalized at the plasma membrane and within small cytoplasmic granules (Fig. 5.7). In quail fibroblast (QT6) cells expressing RSV Gag-GFP, however, myc-Mov10 was diffusely cytoplasmic while RSV Gag-GFP localized predominantly to the plasma membrane, indicating that RSV Gag does not strongly colocalize with Human
Figure 5.7 Gag proteins from diverse retroviruses colocalize with Mov10. NMuMG cells expressing MMTV Gag-GFP or MLV Gag-GFP, HeLa cells expressing HIV-1 Gag-GFP, or QT6 cells expressing RSV Gag-GFP were co-transfected with myc-Mov10 and immunostained using an anti-myc antibody.
Mov10. Taken as a whole, these data indicate that Mov10 localizes to sites of viral replication for diverse mammalian retroviruses.

5.4 Discussion

The role that P-bodies and RISCs and their associated proteins play in retroviral replication is only beginning to be understood. Recent evidence has suggested that HIV-1 may interact with some P-body and RISC proteins, including Mov10, Ddx6, and Ago2, to facilitate viral replication [319,320,329]. However, on the other hand, evidence suggests that Mov10 also inhibits the replication of HIV-1 and other retroviruses, as well as endogenous retrotransposons [326,327,328,330]. Furthermore, other evidence indicates depletion of Ddx6, Ago2, and indeed P-bodies in general increases HIV-1 infectivity [336]. When viewed as a whole, these findings suggest that P-bodies and RISCs may play a conserved role in limiting the replication of retroviruses and retrotransposons, but that some viruses have evolved mechanisms to circumvent or hijack these cellular defenses.

Interestingly, we found that MMTV Gag colocalized with endogenous Mov10 at intracytoplasmic sites of virus assembly (Fig. 5.1A). Interestingly, Gag did not colocalize with Ddx6, but did colocalize with cytoplasmic structures stained with the human serum 18033, which contains antibodies to both Ge-1 (P-bodies) and GW-182 (P-bodies and RISCs) (Fig. 5.1) [410]. Together, these observations suggest that MMTV capsid assembly does not occur in conjunction with P-bodies
per se, but that MMTV assembly complexes may contain P-body and/or RISC proteins.

In support of this idea, overexpression of Mov10, Ago1, or Ago2 caused Gag to accumulate in large complexes containing P-body or RISC proteins while Gag localization was unaffected by RFP-Dcp1a expression (Fig. 5.2). Interestingly, however, we did observe that foci of MMTV Gag were frequently adjacent to and partially overlapping RFP-Dcp1a foci (Fig. 5.2, enlargement). Similar “docking” interactions have been reported between SGs and P-bodies, and are thought to mediate transfer of mRNAs between the two structures [274]. Accordingly, we hypothesize that MMTV RNA may transiently localize to P-bodies, where it is bound by Gag.

Indeed, using live-cell RNA imaging techniques we observed that MMTV RNA transiently trafficked to RFP-Dcp1a-containing P-bodies in the absence of Gag, indicating that Gag is not required for viral RNA to traffic to P-bodies (Fig. 5.5). However, the precise mechanism by which the RNA is targeted to P-bodies remains unclear. Cellular miRNAs target cellular mRNA transcripts to RISCs, so one possibility is that MMTV RNA could be targeted to P-bodies and/or RISCs by a cellular or viral-encoded miRNA [303]. Alternatively, a cis-acting sequence within the viral RNA, such as an AU-rich element (ARE) could also promote trafficking of the RNA to P-bodies [300]. However, these ideas remain to be tested.

Conversely, Gag may also traffic to P-bodies independently of RNA, because MMTV Gag truncation mutants lacking NC, which is required for both
Gag-RNA and Gag-Gag interactions, accumulate in P-bodies when P-body proteins are overexpressed (Fig. 5.3). However, RNA does appear to be required for the formation of a complex between Gag and Mov10 (Fig. 5.4), suggesting a domain of Gag other that NC targets Gag to P-bodies, but that RNA, and likely NC, are required for Gag to interact with P-body proteins. However, we cannot conclusively rule out the possibility that another domain of Gag, such as MA, may participate in RNA-dependent interactions with P-body proteins [412,413].

We were surprised to observe that even though overexpression of Mov10 causes a dramatic change in MMTV Gag localization, there was no concomitant effect on MMTV virus production, while knockdown of Mov10 caused a significant decrease in MMTV particle production (Fig. 5.6). These data suggest that MMTV has evolved to hijack Mov10 to facilitate its own replication, however we did not address the role of Mov10 in MMTV infectivity. Interestingly, Mov10 also localized to assembly sites of several other mammalian retroviruses, including MLV and HIV-1, suggesting that Mov10 may act to promote or inhibit the replication of many mammalian retroviruses.
Chapter 6

Alterations in the MA and NC Domains Modulate Phosphoinositide-Dependent Plasma Membrane Localization of the Rous Sarcoma Virus Gag Protein

Darrin V. Bann*, Shorena Nadaraia-Hoke*, Timothy L. Lochmann, Nicole Gudleski-O'Regan, and Leslie J. Parent
*Authors contributed equally


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Abstract

Retroviral Gag proteins direct virus particle assembly from the plasma membrane (PM). Phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2] plays a role in PM targeting of several retroviral Gag proteins. Here we report that depletion of intracellular PI(4,5)P2 and phosphatidylinositol-(3,4,5)-triphosphate [PI(3,4,5)P3] levels impaired Rous sarcoma virus (RSV) Gag PM localization. Gag mutants deficient in nuclear trafficking were less sensitive to reduction of intracellular PI(4,5)P2 and phosphatidylinositol-(3,4,5)-triphosphate [PI(3,4,5)P3] levels, suggesting a possible connection between Gag nuclear trafficking and phosphoinositide-dependent PM targeting.

*Please see Appendix B for a reprint of the journal article comprising this data chapter.
Chapter 7

Overall Discussion
7.1 Introduction

Perhaps one of the most fascinating aspects of retroviruses is that these viruses must carry out all of the functions required to replicate using a very limited genome. Additionally, retroviruses must also avoid or inactivate cellular defensive pathways that have evolved to limit the replication of both exogenous retroviruses and endogenous retrotransposons. As a result, retroviruses interact with a multitude of cellular factors both to directly facilitate virus replication and to create a cellular environment that is permissive for virus production. The data presented in this dissertation have demonstrated that MMTV and RSV interact with multiple cellular factors to facilitate several stages of viral replication, including intracellular RNA trafficking, particle assembly, and plasma membrane targeting. This chapter will attempt to address the implications of these interactions, and how these findings contribute to the understanding of retroviral replication and cellular biology in general.

7.2 Studying MMTV replication in the context of chronic infection

An important consideration in the interpretation of many of the MMTV experiments conducted in this dissertation is that the NMuMG-C3H cell line used for these experiments is chronically infected with MMTV. Because MMTV does not create a barrier to superinfection [55], these cells are likely to contain multiple integrated MMTV proviruses in different locations throughout the genome. Accordingly, these cells express high levels of MMTV in the absence of dexamethasone or other steroid hormones normally used to induce MMTV
expression. That this high-level virus expression is not cytotoxic suggests that these cells may have undergone adaptive changes in culture to balance the requirements of high-level virus production with the requirements of physiological cellular processes.

Based on these observations, it is not unreasonable to conclude that within NMuMG-C3H cells there exists a dynamic equilibrium between viral factors that promote virus replication and cellular factors that inhibit virus replication to prevent overwhelming damage to the cell. The disadvantage to this system is that we cannot observe the acute changes to cellular physiology that occur in conjunction with retrovirus infection. Accordingly, the results described in this dissertation may under-report changes to cellular physiology resulting from MMTV infection. On the other hand, the equilibrium between viral and cellular processes in NMuMG-C3H cells can be advantageous because the equilibrium can be manipulated to produce a phenotypic change, which can be studied.

7.3 Nuclear & nucleolar trafficking of Gag

Gag proteins from several retroviruses, including RSV, HIV-1, MLV, FIV, and Foamy virus, localize to the nucleus [94,251,414,415,416,417,418]. Here, we report that a sub-population of MMTV Gag also localizes to the nucleus and nucleoli under steady-state conditions (Appendix A Figure 1). The observation that Gag proteins from viruses spanning five genera of Retroviridae localize to the nucleus suggests that nuclear trafficking of Gag plays a conserved role in retroviral replication. However, while genetic evidence suggests that nuclear
trafficking of RSV Gag is linked to genome packaging [95], the role of nuclear Gag trafficking in the replication of other retroviruses, including MMTV, is not known.

One possibility is that like RSV, MMTV Gag may traffic through the nucleus to bind genomes for encapsidation. This model is attractive because it would allow for co-transcriptional encapsidation of viral RNAs, thereby eliminating the need for Gag to locate genomes available for encapsidation in the cytoplasm. Furthermore, nuclear encapsidation of genomes would provide an elegant solution to the problem of sorting viral RNAs for use as mRNA or genomes: RNAs bound by Gag in the nucleus would be exported and encapsidated, while RNAs exported from the nucleus through other means, such as Rem, would be used for translation.

Our observation that MMTV Gag accumulates in nucleoli, together with data from MMTV and other retroviruses, may support a model where Gag traffics through the nucleus to bind viral RNA. MMTV Rem and analogous proteins in HIV-1 (Rev), HTLV-1 (Rex), and JSRV (Rej) localize predominantly to the nucleolus [19,20,238,244,419,420], suggesting that nuclear export of unspliced retroviral RNAs may involve a nucleolar trafficking step. Additionally, HIV-1 RNA is degraded by ribozymes targeted to the nucleolus, indicating that retroviral RNA is present within nucleoli [241]. Therefore, it is relatively easy to envision that MMTV Gag may traffic through nucleoli for the purpose of packaging viral RNA. However, evidence from HIV also suggests that Rev is required for efficient RNA encapsidation, indicating that trafficking of HIV-1 Gag through the nucleus is not sufficient to package viral genomes [151,392]. Accordingly, further
experimentation is required to determine whether nuclear accumulation of MMTV Gag is related to RNA encapsidation or another function.

However, the experiments required to demonstrate a role of nuclear trafficking in MMTV genomic RNA encapsidation may prove difficult. As far as we know, MMTV has only one nuclear localization signal and/or nucleolar localization signal within NC, as the NC domain of Gag localizes to nucleoli when expressed alone [421]. Accordingly, genetic mutation of the putative NLS/NoLS may also disrupt the ability of NC to bind RNA in general. In light of this obstacle, a better approach may be to reduce nuclear trafficking of Gag by inserting a strong exogenous nuclear export signal into Gag. While this mutation would not ablate nuclear trafficking it may cause Gag to traffic through the nucleus more quickly or may disrupt the subnuclear localization of Gag. Either outcome could therefore have the potential to reduce genomic RNA packaging if nuclear and/or nucleolar trafficking of Gag is required for encapsidation. An alternative approach could be to disrupt nuclear trafficking of MMTV Gag by strongly targeting the protein to the plasma membrane, similar to what has been done previously in Rous sarcoma virus [95]. Although MMTV Gag is already myristoylated (in contrast to RSV, which is acetylated), the addition of the Src membrane binding domain to the N-terminus of MMTV Gag may be sufficient to target Gag strongly to the membrane. However, this approach may be problematic because MMTV assembles in the cytoplasm, not at the plasma membrane, so disrupting the cytoplasmic trafficking of Gag may also reduce genomic RNA encapsidation independently of the effect on Gag nuclear trafficking.
On the other hand, nucleolar localization of Gag may also be involved in MMTV pathogenesis. The nucleolus controls many cellular functions including cell growth and apoptosis. Recent reports show that the nucleolar protein Pict1 acts to retain RPL11 in the nucleolus, preventing RPL11 from disrupting the cytoplasmic interaction between MDM2 and p53, resulting in increased tumor growth and progression [223,224]. Although RPL9 has not been previously implicated in p53 regulation, one possibility is that MMTV Gag may interact with RPL9 in nucleoli to prevent translocation of free RPL9 into the cytoplasm where it could release p53 from MDM2-mediated degradation. However, several steps would be required to establish a connection between Gag and the regulation of p53 stability by RPL9.

The first step would be to determine whether RPL9 does, in fact, play a role in p53 regulation. Several ribosomal proteins, including RPL5, RPL11, RPL23, and RPS7 directly interact with MDM2 to inhibit ubiquitination and degradation of p53 [229,230,231,422,423]. Accordingly, co-immunoprecipitation experiments could be used to determine whether RPL9 also forms a complex with MDM2. However, other ribosomal proteins including RPL29 and RPL30 influence p53 stabilization without directly interacting with MDM2 [424], so a negative co-immunoprecipitation result between RPL9 and MDM2 could not be interpreted to mean that RPL9 does not influence p53 regulation.

A more general measure of whether RPL9 influences p53 stability could be accomplished by knocking down RPL9 expression and measuring p53 levels by Western blotting. Although perturbations in ribosomal protein expression can
generally result in p53 accumulation [425], siRNA knockdowns have been used successfully to demonstrate the role of specific ribosomal proteins in p53 regulation [232,424,426]. Alternatively, co-transfection of exogenous p53 and RPL9 could be used to test whether RPL9 influences the stability of exogenous p53 [229].

To evaluate the role of Gag in modulating RPL9-mediated regulation of p53, experiments would have to be conducted in cells that are not infected with MMTV because MMTV Env modulates p53 stabilization, which would confound any effects of Gag on p53 [427]. Accordingly, plasmids encoding MMTV Gag or Gag mutants could be used to determine the effect of Gag on a potential RPL9-MDM2 interaction by co-immunoprecipitation. Furthermore, this approach could be used to test whether Gag expression is sufficient to reduce the accumulation of p53 in response to cellular stress, such as serum starvation or actinomycin D treatment.

7.4 Extraribosomal functions of RPL9

Ribosomal proteins play critical roles in ribosomal function, however a growing body of literature indicates that ribosomal proteins also have other functions outside the context of the ribosome. For example, several ribosomal proteins including RPL5, RPL11, RPL23, and RPS7, regulate intracellular p53 levels by interacting with MDM2 [229,230,231,232,426,428]. Alternatively, other ribosomal proteins regulate splicing and/or stability of their own mRNA, while still others have roles in cell signaling, antiviral defense, and transcriptional regulation.
(reviewed in [429]). With regards to retroviruses, overexpression of RPL4 was recently found to enhance readthrough of the MLV gag-pol junction by twofold, which resulted in defective particle assembly [430]. Because ribosomal proteins are synthesized far in excess of what is needed for ribosome assembly under steady-state conditions [431], these data suggest that RPL4 acts outside of the ribosome to influence MLV Gag-Pol translation.

Similarly, our data indicate an extraribosomal role for RPL9 in MMTV replication. Specifically, knockdown of RPL9 decreased the production of MMTV virus particles, however we detected no change in the Gag:Gag-Pro:Gag-Pro-Pol ratio by Western blotting (Appendix A Fig. 8), suggesting that RPL9 does not inhibit virus production by increasing the rate of ribosomal frameshifting. Due to the short span of RPL9 knockdown (36 hours), the level of RPL9 in cytosolic ribosomes is unlikely to be affected because ribosomes are disassembled as a functional unit with a $t_{1/2}$ of 96 – 120 hours [432,433]. Indeed, in cells treated with the RPL9 siRNA and immunostained for RPL9, we observed a decrease in nucleolar fluorescence, but not cytoplasmic fluorescence, compared to cells treated with scrambled control siRNA, indicating that the knockdown affected only RPL9 that was not yet incorporated into ribosomes (Appendix A Fig. 8). As a result, these data indicate that RPL9 influences the production of MMTV particles through a mechanism independent of the role RPL9 plays in the ribosome.

However, the mechanism by which RPL9 influences MMTV replication remains unknown. Structural and biochemical evidence based on the bacterial ortholog of RPL9, (confusingly) called RPL6, indicates that RPL6 binds the
prokaryotic 23S rRNA, potentially to stabilize the rRNA secondary structure [434,435]. Specifically, the C-terminal domain of RPL6 interacts with the 23S rRNA, while the N-terminal domain seems to participate in protein-protein interactions [435,436]. Surprisingly, we found that multiple domains of Gag interact directly or indirectly with the C-terminal domain of eukaryotic RPL9 (Appendix A Figs. 4, 5 & 7; data not shown), raising the possibility that RPL9 could bind a specific secondary structure within the viral RNA and recruit Gag to the RNA through a protein-protein interaction. As a result, reducing the availability of extraribosomal RPL9 may reduce recruitment of Gag to the viral RNA.

One way to test this hypothesis would be to determine whether RPL9 also localizes to cytoplasmic granules with the subviral RNA described in chapters 4 and 5. Localization of RPL9 to these complexes would suggest that RPL9 binds the viral RNA, potentially to recruit Gag. To test whether RPL9 is required to recruit Gag to these complexes, extraribosomal RPL9 levels could be reduced using siRNA, and confocal microscopy could be used to determine whether Gag is still able to accumulate in svRNA granules in the absence of RPL9. To ensure that any observed phenotype is specific to the depletion of RPL9, endogenous RPL9 levels could be repleted using an siRNA-resistant RPL9 construct.

Alternatively, RPL9 could have a similar function to RPL4 in MLV, and facilitate frameshifting events needed to produce Gag-Pro and Gag-Pro-Pol [430]. Although we did not detect an increase in frame shifting by Western blotting (Appendix A Fig. 8), a decrease in frameshifting may be difficult to detect by this
technique due to low expression of Gag-Pro and Gag-Pro-Pol. In our hands, MMTV Gag is not sufficient to produce virus like particles (data not shown), suggesting that other components, including \( \Psi \)-containing RNA, Gag-Pro, and/or Gag-Pro-Pol, may be required. As a result, if knockdown of RPL9 decreases the frequency of ribosomal frameshifting the ratio of Gag:Gag-Pro:Gag-Pro-Pol may be disturbed, resulting in a budding defect.

However, the budding defect observed during RPL9 knockdown may also be a non-specific effect. Although we did not observe any overt toxicity from RPL9 knockdown during the course of the experiment, we did find that RPL9 knockdown increased p53 accumulation by almost 2.5-fold relative to cells treated with scrambled siRNA (data not shown). We do not know whether this increase is a direct result from RPL9 depletion, or whether this increase was an off-target effect resulting from an impairment of ribosome biogenesis [424,437]. Furthermore, the functional implications this increase are unknown. We did not observe any effect of RPL9 knockdown on steady-state Gag or GAPDH levels, suggesting that ribosomal function was not affected, and cells in which RPL9 was knocked down appeared morphologically normal by immunofluorescence confocal microscopy (Appendix A Fig. 8). Furthermore, MMTV inhibits apoptosis through an immunoreceptor tyrosine-based activation motif (ITAM)-mediated pathway, suggesting that MMTV infection may limit the effects of increased p53 levels [438]. As a result, it may be unlikely that the effect of RPL9 knockdown on MMTV budding is due simply to off-target cytotoxic effects.
7.5 Stress granules, P-bodies, and retroviral replication

In addition to the rRNA-binding protein RPL9, we found that MMTV interacts with a number of cellular mRNA binding proteins associated with SGs, P-bodies, and RISCs. We observed colocalization between Gag and certain endogenous SG and P-body proteins, such as YB1 and Mov10, but only limited colocalization of Gag with other SG or P-body proteins, including TIA1 and Ddx6 (Chapter 3 Fig. 3.1; Chapter 5 Fig. 5.1). Furthermore, functional assays examining MMTV virus production supported the colocalization findings in that knockdown of YB1 or Mov10 inhibited virus production, while TIA1 knockdown had no effect on production (Chapter 3 Fig. 3.8; Chapter 5 Fig. 5.6). Together, these data indicate that while the MMTV assembly pathway interacts with specific SG and P-body proteins, assembly does not occur in conjunction with SGs or P-bodies per se. Such a model is not unprecedented in the literature. For example, while replication of the yeast retrotransposon Ty3 is dependent on P-body proteins, Ty3 replication is thought to occur in complexes called “retrosomes”, which are independent of P-bodies [171]. Similarly, metazoan retroviruses including HIV-1 and foamy virus (FV) interact with a variety of SG (i.e. Staufen-1), P-body (i.e. Ddx6), and RISC (i.e. Ago2) proteins, however there is no evidence that these viruses interact directly with SGs, PBs, or RISCs to facilitate virus assembly [147,148,319,320,439]. As a result, multiple, diverse retroviruses have evolved to hijack cellular mRNA binding proteins to facilitate virus replication.

As a result, we were surprised to find that MMTV Gag accumulated in SGs, P-bodies, or RISCs induced by heat shock or overexpression of several proteins.
including YB1, TIA1, Mov10, and Ago2 (Chapter 3 Figs. 1 & 2; Chapter 5 Fig. 2). However, this dramatic re-distribution of Gag in response to TIA1 or Mov10 overexpression did not affect virus production, suggesting that SGs or P-bodies per se do not inhibit MMTV virus production (Chapter 3 Fig. 8, Chapter 5 Fig. 6).

By contrast, we found that knocking down YB1 inhibited virus production, while knockdown of TIA1 had no effect on virion production, suggesting that specific SG-associated proteins influence MMTV virus assembly, but that assembly does not depend on SGs per se.

In support of the observation that Gag interacts with specific SG proteins to facilitate assembly, we found that Gag traffics rapidly in and out of SGs induced by overexpression of G3BP1, but is less dynamic in SGs induced by overexpression of YB1. The observation that FRAP of Gag within YB1-induced SGs is modeled by a single exponential equation and that Gag exhibits only limited recovery after bleaching suggests that Gag is bound to a single partner within YB1-containing SGs, potentially the viral RNA. Indeed, our finding that Gag interacts with YB1 through an RNA-mediated mechanism (Chapter 3) and that YB1 colocalizes with viral RNA in the absence of Gag (Chapter 4 Fig. 4.2) suggests a model where YB1 binds specific sequences within the viral RNA, then recruits Gag to bind the RNA for encapsidation. One way to test this model would be to determine whether Gag is still recruited to viral RNA when YB1 is knocked down. However, because YB1 is essential for translation it may be difficult to sufficiently reduce YB1 levels to see a dramatic effect on Gag localization. Alternatively, the ability of YB1 to recruit Gag to specific transcripts could be
tested by tethering YB1 to a non-viral transcript that does not normally recruit Gag using MS2 stem-loops and a chimeric protein where YB1 is fused to the MS2 coat protein (for examples, see [440]). Thus, recruitment of Gag to the MS2 stem loop-containing RNA in the present of a YB1-MS2 chimera would indicate that YB1 is the major cellular protein required to recruit Gag to viral RNAs.

We were also interested to find that MMTV neither promotes nor inhibits SG formation during chronic infection. Several cytotoxic RNA viruses induce SG formation to inhibit cellular protein synthesis in favor of the synthesis of viral proteins [309,310,311,381]. However, while retroviruses such as HIV-1 may affect cellular protein synthesis by protease-mediated cleavage of cellular translation initiation factors [441,442], retroviruses have not been reported to induce SGs to mediate shutoff of cellular protein synthesis. By contrast, HIV-1 and HTLV-1 have been reported to inhibit SG formation [148,314], so we were surprised to find that MMTV infection did not affect SG induction by two different stimuli: heat shock and overexpression of SG proteins (Chapter 3 Figs. 1 & 2). SGs, P-bodies, and RISCs play an important role in antiviral defense, and many viruses inhibit SG formation to promote viral replication. Accordingly, the observation that MMTV does not inhibit the formation of SGs suggests that MMTV has evolved other mechanisms to overcome this cellular defensive pathway.

If SGs, PBs, and RISCs represent a cellular antiviral defense, then why do MMTV Gag and RNA accumulate within these complexes? One possibility is that MMTV has adapted to this cellular defense by targeting Gag to these complexes to protect the viral RNA from degradation. As a result, SGs, PBs, and/or RISCs
may represent an intracellular “tug-of-war” over the viral RNA, with viral factors
trying to promote viral replication on one side, and cellular factors trying to restrict
viral replication on the other. However, given our observation that overexpressing
SG or P-body proteins does not affect MMTV virus production, the targeting of
MMTV Gag to these structures may efficiently disable any antiviral effects.

Accordingly, one might expect that viral RNA expressed in the absence of
Gag (i.e. a non-translatable RNA) would be relatively unstable, but that the
addition of Gag in trans would have a stabilizing effect on the viral RNA. Because
MMTV Gag by itself is not sufficient to produce virus particles, this experiment
would be relatively straight-forward since the experiment would not have to
account for viral RNA encapsidated into particles and released from the cell. To
run the experiment, uninfected NMuMG cells would be transfected with the non-
translatable viral RNA with or without Gag, and cell lysates would be collected.
The stability of the RNA in the cell lysate in the absence or presence of Gag could
then be assessed by quantitative real-time PCR or ribonuclease protection assay.

However, we have not examined the effect of SG or P-body induction on
MMTV gRNA packaging, so it remains a possibility that viruses produced during
SG induction are non-infectious. Cell-free MMTV is poorly infectious, so this
possibility is somewhat difficult to test, however co-culture infectivity assays have
been used to study HTLV-1 infectivity. Accordingly, MMTV-infected cells could be
transfected with SG proteins then labeled with a lipophilic fluorescent membrane
dye. The cells could then be co-cultured with uninfected cells, and the spread of
infection could be assayed by flow cytometry for the presence of Gag in the
absence of the membrane dye. Conversely, a more direct method to measure the effect of Gag on RNA encapsidation would be to quantify the amount of viral RNA incorporated into virus particles released from the cell using a ribonuclease protection assay or quantitative reverse-transcription PCR.

However, other retrovirus-like elements may target Gag-like proteins to SGs, P-bodies, and/or RISCs to evade cellular defensive pathways. For example, the endogenous long interspersed nuclear element (LINE)-1 retrotransposon induces the formation of large RNPs containing SG, PB, and RISC proteins, as well as LINE-1 RNA, which serves as a genome, and the LINE-1 proteins Orf1p and Orf2p [323,382]. These LINE-1 RNPs are thought to represent replication intermediates, however other data suggests that they may also represent a cellular attempt to restrict LINE-1 replication [326,330,411]. Amazingly, we found that MMTV Gag colocalizes with a set of SG, P-body, and RISC proteins identical to the set observed to colocalize with Orf1p, which is functionally analogous to Gag [323,382]. This suggests that either a conserved cellular pathway acts on both exogenous retroviruses and endogenous retrotransposons, or alternatively that the evolution of MMTV and LINE-1 has converged on a particular cellular pathway to facilitate both retrovirus replication and retrotransposition.

7.6 RNA Trafficking to SGs and P-bodies

During cellular stress many mRNAs encoding “housekeeping” genes are translationally repressed in SGs, while other mRNAs, such as those encoding heat shock proteins are selectively excluded from SGs [269]. However, the
mechanism that regulates trafficking of specific transcripts to SGs remains unclear. By contrast, the mechanisms implicated in targeting mRNAs to P-bodies are somewhat better defined. Specifically, AREs in the 3’ UTR and other regions of the transcript promote transcript destabilization and targeting to P-bodies [300]. Furthermore, miRNAs may also target RNAs to P-bodies for translational repression and/or degradation [303]. We found that MMTV sub-viral RNA was sufficient to form SG-like structures with SG proteins and transiently co-localize with P-body proteins in the presence of Rem (Figs. 4.3, 5.5), suggesting that cis- or trans-acting signals may target MMTV RNA to SGs and/or P-bodies.

Unfortunately, the signals responsible for targeting MMTV RNA to these granules (or, alternatively, promoting the formation of SG-like granules on the viral RNA) remain unclear. As discussed above, the MMTV svRNA construct does contain several AUUUA pentamers, which are bound by a variety of SG-associated proteins including YB1, HuR, and TTP [400]. Accordingly, one possibility is that some or all of these proteins could bind AUUUA pentamers in the svRNA, thereby targeting the transcript to SGs and/or P-bodies. Interestingly, the precise sequence of AREs can significantly influence the effect of the ARE on transcript stability. For example, multiple tandem AUUUA pentamers in the interleukin-8 (IL-8) and granulocyte/macrophage-colony stimulating factor (GM-CSF) mRNAs promote degradation, while two separated AUUUA pentamers in the GM-CSF mRNA are bound by HuR and promote transcript stabilization [443]. Moreover, YB1 also binds AREs to help stabilize mRNA transcripts [397,444], which may indicate that YB1 colocalizes with the MMTV RNA to stabilize the virus
genome and prevent degradation. As a result, the presence of dispersed AUUUA repeats in MMTV RNA may be an evolutionary adaptation by the virus to facilitate binding of the genome by cellular proteins that promote RNA stabilization. This hypothesis could simply be tested by mutating the AU-rich elements within the subviral RNA and observing whether this RNA still recruited SG proteins to cytoplasmic granules. Furthermore, if the AU-rich elements exert a stabilizing effect on the viral RNA, mutation of these elements would be expected to result in increased viral RNA degradation. As a complementary approach, the effect of dispersed AU-rich elements on the recruitment of SG proteins and RNA stability could be tested by inserting AUUUA sequences into heterologous, non-viral RNAs, and testing whether these RNAs now interacted with SG-associated proteins such as HuR, and whether the transcripts were more stable over time.

On the other hand, trafficking of MMTV RNA to SG-like complexes may represent an attempt by the cell to translationally repress or degrade viral RNAs. Many SG-associated proteins, including YB1, TTP, and G3BP1 have antiviral functions [279,286,287], so these proteins may interact with viral RNA as part of an intrinsic cellular defense pathway. Inhibition of the miRNA pathway by knocking down the miRNA processing molecules Dicer and Drosha increases HIV-1 protein expression and infectivity [445], suggesting that miRNA-mediated gene silencing may also play an important role in cellular defense against retroviral infection. As a result, it is possible that endogenous murine miRNAs may bind MMTV RNA and target the transcript to SGs and/or PBs for translational repression and/or degradation. However, recent evidence also indicates that a
specific miRNA, miR-132, actually enhances HIV-1 replication, suggesting that retroviruses may have evolved to use the miRNA pathway to their advantage [446]. Further evidence that miRNAs may aid retroviral replication is provided by the observation that retroviruses may actually encode their own miRNAs [447].

Most cellular miRNAs are located in intergenic regions or in introns of pre-mRNAs, and therefore may not have significant complementarity to the mature transcript [448] however a retroviral miRNA would likely have significant complementarity to its own genomic RNA. As a result, an intriguing but yet untested hypothesis is that MMTV may encode a self-targeting miRNA to tag a portion of its full-length RNA for translational repression and encapsidation into virus particles as a genome.

7.7 Cytoplasmic role of Rem in MMTV Replication

In addition to cis-acting sequences within the viral RNA, our data indicate that the trans-acting factor Rem may also influence cytoplasmic trafficking of viral RNA. We found that when Rem-mCherry was expressed in uninfected NMuMG cells, MMTV RNA was exported from the nucleus, as expected, but Rem accumulated with the RNA in cytoplasmic granules containing YB1 (Chapter 4 Fig. 3). This finding is particularly interesting because all of the functional motifs of Rem are found in the SP domain, and previous reports have indicated that SP must be proteolytically cleaved from full-length Rem to function as an RNA export protein [83,85]. By contrast, because our Rem-mCherry protein is C-terminally tagged with a fluorophore, our results suggest that full-length Rem is colocalized
with viral RNA in the cytoplasm. This observation was confirmed by immunostaining with an anti-Rem/SP antibody, which demonstrated the presence of SP within these complexes (data not shown). However, we cannot exclude the possibility that SP is first cleaved from the ΔSUΔTM-mCherry domain, traffics into the nucleus, exports viral RNA, then re-localizes with the ΔSUΔTM-mCherry domain.

Interestingly, cytoplasmic colocalization of Rem or other Rev-like proteins with retroviral RNA has not been previously reported. However, evidence from HIV-1 does suggest that Rev does play an important role in the cytoplasmic fate of viral RNA. For example, Rev increases translation of unspliced HIV-1 RNAs independently from the role of Rev in unspliced RNA export from the nucleus [389,391], and furthermore Rev may also influence the selection of genomes for packaging [150,151,392]. As a result, Rem may play an important role in the encapsidation or translation of MMTV RNA. Our observation that Rem colocalizes in cytoplasmic complexes with viral RNA and YB1 suggests that Rem may help traffic MMTV RNA to cytoplasmic sites of capsid assembly. However, the precise role that Rem plays in MMTV RNA trafficking and/or encapsidation remains to be determined.

Thankfully, the experiments to test the role of Rem in MMTV RNA trafficking should be relatively straightforward. HIV-1 Rev and HTLV-1 Rex can both substitute for Rem to export RemRE-containing RNA from the nucleus [88], however because both HIV1- and HTLV-1 assemble virus particles at the plasma membrane, Rev or Rex may not target RNAs to the same subcellular location as
Rem. Accordingly, Rev or Rex could be expressed with the MMTV subviral RNA construct to determine whether Rev or Rex cause aberrant targeting of the viral RNA. Furthermore, if this RNA were to be expressed in the context of virus-infected cells, RNA packaging assays could be used to determine whether Rev or Rex fail to target a Ψ-containing RNA for encapsidation. Similarly, the requirement for a trans-acting Rev-like protein could be ablated entirely by replacing the RemRE with the constitutive transport element from MPMV, which would test whether Rev-like proteins are required for proper localization and encapsidation of MMTV RNA. Alternatively, as a gain-of-function approach the RemRE could be inserted into a non-viral RNA labeled with BoxB stem-loops and λN-eGFP. In this system, if Rem is responsible for the localization of MMTV RNA to cytoplasmic granules, the expression of Rem would then cause the non-viral RNA to accumulate in cytoplasmic granules containing YB1 and other SG-associated proteins.

It should be noted, however, that our observed localization of Rem could be an artifact of overexpression. During infection, Rem is likely to be expressed at relatively low levels because it is translated from a doubly spliced RNA. As a result, expression of Rem from a construct driven by the CMV promoter may result in vastly higher Rem expression than is ever observed during infection. So, it is possible that under these conditions, free Rem in the cytoplasm may bind viral RNA without contributing directly to viral RNA localization. Unfortunately, visualization of viral Rem expressed during infection is complicated by the presence of SP on both Rem and Env proteins [83], making it impossible to
differentiate Rem from Env using the anti-Rem/SP antibody. As a result, further studies of MMTV Rem-mediated RNA trafficking may require the development of a plasmid that expresses tagged Rem at wild-type levels. Perhaps the easiest way to accomplish this would be to replace the CMV promoter on a Rem expression plasmid with a weaker promoter, such as PolII. From this point, additional mutations could be made to the promoter structure to further reduce Rem expression to levels observed during virus infection.

7.8 Membrane targeting of retroviral Gag proteins

To be released from the host cell, Gag proteins from all retroviruses must eventually traffic to the plasma membrane (PM). The MA domain of Gag mediates membrane trafficking, however membrane binding must be specific for the plasma membrane to avoid the accumulation of Gag on intracellular membranes. Accordingly, the MA domains of many retroviral Gag proteins interact with the acidic, PM-specific phospholipid phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P$_2$] to promote a conformational change in Gag, which allows for membrane binding [206,449,450]. In contrast to previous studies [451], we found that PM localization of Rous sarcoma virus Gag was sensitive to enzymatic depletion of PI(4,5)P$_2$ and PI(3,4,5)P$_3$ [Phosphatidylinositol-(3,4,5)-triphosphate] (Chapter 6). Our most interesting finding, however, was that two RSV Gag mutants, Myr1E.Gag and Gag.M1, which undergo reduced nuclear trafficking, were also less sensitive to PI(4,5)P$_2$ and PI(3,4,5)P$_3$ depletion, thereby establishing a link between nuclear trafficking and PM targeting of RSV Gag.
Interestingly, membrane-free (or at least detergent-resistant) PI(4,5)P$_2$ is present in the nucleus, where it is implicated in protein localization and signaling cascades [452,453,454,455]. As a result, we hypothesize that RSV Gag may interact with PI(4,5)P$_2$ in the nucleus and not necessarily at the PM. However, testing this hypothesis may prove to be challenging because it may be difficult to reduce nuclear PI(4,5)P$_2$ levels without affecting cytoplasmic PI(4,5)P$_2$. One potential approach could be to add a heterologous nuclear localization signal to 5PtaseIV, which would target the enzyme to the nucleus, thereby depleting nuclear PI(4,5)P$_2$. However, given the important role of PI(4,5)P$_2$ in the nucleus, depletion of PI(4,5)P$_2$ from the nucleus may be toxic.

Puzzlingly, the nucleus seems to contain all of the components necessary for capsid assembly, including Gag, RNA, and PI(4,5)P$_2$, however capsid assembly does not seem to occur within the nucleus even when Gag is trapped by mutating its nuclear export signal. As a result, we have frequently wondered about the mechanism that prevents capsid assembly in the nucleus, and surprisingly the answer may come in the form of PI(4,5)P$_2$. Unpublished in vitro data from our lab examining assembly of Gag into virus-like particles (VLPs) found that Gag did not assemble VLPs in the presence of PI(4,5)P$_2$, suggesting that in contrast to other retroviruses, PI(4,5)P$_2$ may actually function to prevent assembly of Gag on inappropriate cellular membranes. As a result, depletion of PI(4,5)P$_2$ may cause RSV Gag to assemble capsids on intracellular membranes, rather than at the PM.
The mechanisms involved in targeting MMTV to membranes are less clear. PM targeting of M-PMV capsids, which like MMTV are assembled in the cytoplasm, is mediated by PI(4,5)P_2 [450,456]. However, in contrast to M-PMV, electron microscopy studies of MMTV-infected cells reveal that MMTV frequently buds into intracellular vesicles or multivesicular bodies (our unpublished observations) [160,199]. However, the mechanism used by Gag to target these structures remains unknown. Recent reports indicate that RISCs are associated with the cytoplasmic face of multivesicular bodies and furthermore, that RISC components are present in endosomes derived from the fusion of multivesicular bodies with the PM [306]. Given our observation that MMTV Gag interacts with RISC components including Ago2 (Chapter 5), one possibility is that Gag could interact with Ago2 to help target assembling virus particles to endosomes or multivesicular bodies. Virus particles could then bud into these structures and be transported to the PM where virus particles could be released with cellular exosomes.

One method to test this model would be to overexpress components of the ESCRT complex, which is required for the formation of multivesicular bodies. When overexpressed, ESCRT components such as Tsg101 exert a dominant-negative effect, which inhibits ESCRT function and the budding of cellular membranes into multivesicular bodies. Therefore, if MMTV virus particles do indeed bud into multivesicular bodies, inhibition of the ESCRT machinery may cause Gag to accumulate on the surface of multivesicular bodies. Furthermore, any cellular proteins used by MMTV to traffic to multivesicular bodies, such as
Mov10 or Ago2, may also accumulate with Gag. Accordingly, the role of Ago2 or Mov10 in targeting Gag to multivesicular bodies could be tested by knocking down Mov10 or Ago2, inhibiting ESCRT function by overexpressing Tsg101, and observing whether Gag still accumulates on the surface of multivesicular bodies. Alternatively, multivesicular bodies can be concentrated by differential gradient centrifugation [306,457]. Therefore, this technique could be used to test whether Gag co-sediments with multivesicular bodies. Furthermore, knocking down specific cellular proteins and assaying Gag co-sedimentation with multivesicular bodies could be used to test the role of cellular proteins in targeting Gag to multivesicular bodies. Therefore, if a cellular protein is required for Gag localization to MVBs, it would be expected that knocking down that protein would reduce cosedimentation of Gag with MVBs.

7.9 Conclusions and Overall Model of MMTV Assembly

The data presented within this dissertation provide insight into the molecular mechanisms underlying the assembly of MMTV and RSV. We propose a model of MMTV assembly where full-length viral RNA is exported from the nucleus by Rem. A portion of this RNA is used to direct translation of Gag, Gag-Pro, and Gag-Pro-Pol, but another portion of RNA is targeted to SG-like viral RNPs (vRNPs) by cis- or trans-acting factors, where the viral RNA is translationally repressed. Gag is then able to traffic to these complexes, where it binds the RNA as a genome. We then propose that components of the vRNP associated with RISCs help target the assembling virus particles to multivesicular
bodies or endosomes where capsid assembly is completed. Finally, virus particles may either bud into MVBs and be released from the cell with exosomes, or alternatively, virus particles may traffic to the PM by interacting with the cytoplasmic face of MVBs, and subsequently bud from the plasma membrane.

However, many questions regarding this model remain to be answered. For example, do cellular mRNA binding proteins actually regulate the use of viral RNA for translation or packaging? Additionally, what cellular or viral factors actually control MMTV RNA trafficking and how is Gag targeted to the same subcellular location? Furthermore, do RISC-associated proteins actually mediate targeting of assembling capsids to intracellular membranes, or do these proteins play other roles in virus assembly that remain to be defined? The colocalization of MMTV Gag with RISC components also suggests that miRNAs are involved in the MMTV assembly pathway. If so, are these miRNAs cellular or viral in origin, and how do they affect the cytoplasmic fate of the viral RNA?

Similarly, the results presented here also raise new questions about the role of cellular phospholipids in RSV assembly. Specifically, does RSV Gag actually interact with PI(4,5)P_2 in the nucleus, and does this interaction prevent premature assembly of RSV capsids on intracellular membranes? If so, is PI(4,5)P_2 released once Gag reaches the plasma membrane? Providing answers to these questions will contribute to an increased understanding of retroviral replication and may identify new pharmacologic targets to inhibit retroviral replication.
Appendix A

Nucleolar Trafficking of the Mouse Mammary Tumor Virus Gag Protein Induced by Interaction with Ribosomal Protein L9

Darrin V. Bann*, Andrea R. Beyer*, Breanna Rice, Ingrid S. Pultz, Melissa Kane, Stephen P. Goff, Tatyana V. Golovkina, and Leslie J. Parent
*Authors contributed equally

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The mouse mammary tumor virus (MMTV) Gag protein directs the assembly in the cytoplasm of immature viral capsids, which subsequently bud from the plasma membranes of infected cells. MMTV Gag localizes to discrete cytoplasmic foci in mouse mammary epithelial cells, consistent with the formation of cytosolic capsids. Unexpectedly, we also observed an accumulation of Gag in the nucleoli of infected cells derived from mammary gland tumors. To detect Gag-interacting proteins that might influence its subcellular localization, a yeast two-hybrid screen was performed. Ribosomal protein L9 (RPL9 or L9), an essential component of the large ribosomal subunit and a putative tumor suppressor, was identified as a Gag binding partner. Overexpression of L9 in cells expressing the MMTV(C3H) provirus resulted in specific, robust accumulation of Gag in nucleoli. Förster resonance energy transfer (FRET) and coimmunoprecipitation analyses demonstrated that Gag and L9 interact within the nucleolus, and the CA domain was the major site of interaction. In addition, the isolated NC domain of Gag localized to the nucleolus, suggesting that it contains a nucleolar localization signal (NoLS). To determine whether L9 plays a role in virus assembly, small interfering RNA (siRNA)-mediated knockdown was performed. Although Gag expression was not reduced with L9 knockdown, interfering RNA (siRNA)-mediated knockdown was performed. Although Gag expression was not reduced with L9 knockdown, virus production was significantly impaired. Thus, our data support the hypothesis that efficient MMTV particle assembly is dependent upon the interaction of Gag and L9 in the nucleoli of infected cells.

Since its discovery as a milk-transmitted agent in the 1930s, the oncogenic retrovirus mouse mammary tumor virus (MMTV) has served as an important model in breast cancer research and immunology (1). However, little is known about the molecular mechanisms that govern MMTV assembly. The 9-kb MMTV RNA genome consists of the common retroviral elements gag, pro, pol, and env, as well as dut (DUTPase) (2), sag (superantigen) (3), and ren (regulator of export of MMTV mRNA) (4, 5). Like all retroviruses, MMTV uses full-length viral RNA to transcribe the viral structural proteins Gag, Gag-Pro, and Gag-Pro-Pol. The Gag protein directs assembly of complete, immature viral capsids in the cytoplasm, which are subsequently transported to the plasma membrane for release by budding.

Unlike acutely transforming retroviruses like Rous sarcoma virus (RSV), MMTV does not carry an oncogene and instead induces tumors primarily by integrating near cellular oncogenes and disrupting their regulation. In addition, the MMTV Gag and Env proteins also promote tumorigenesis independently of the proviral integration site (6, 7). Moreover, differences in pathogenesis between the highly tumorigenic MMTV(CH) strain and the tumor-attenuated MMTV hybrid provirus (HP) strain map to the CA and NC regions of the Gag protein (6), which led us to hypothesize that the Gag proteins from the CH and HP strains might differentially interact with cellular proteins to promote malignant transformation.

The eukaryotic ribosome serves as the catalytic and regulatory center of cellular protein synthesis and is a key player in many aspects of cell and structural biology. It consists of two subunits, the 60S large subunit and the 40S small subunit, which interact noncovalently to mediate the translation of mRNA into polypeptide products. The large subunit contains the 25S, 5.8S, and 5S rRNAs, in addition to more than 45 proteins (8), including RPL9 (L9), a 192-amino-acid protein (9) present in a single copy within the ribosome (10). L9 plays an important role in proper ribosome formation and in normal growth and development. Dro sophila melanogaster mutants carrying one defective rpl9 allele are characterized by stunted growth, reduced viability, and diminished fertility; homozygosity of the mutated gene is lethal (11). Homozygous mutations of rpl9 are also embryonic lethal in zebrafish (12), emphasizing the necessity of L9 for viability and early development. The crystal structure of L6, the prokaryotic equivalent of eukaryotic L9, provides the basis for structural information on the protein, which consists of two domains with nearly identical folds that may have arisen from an ancient gene duplication event (13).

The structure suggests that the N terminus participates in protein-protein interactions at the interface between the large and small ribosomal subunits, while the C-terminal hydrophobic residues bind the 28S rRNA within the interior of the ribosome (13). In the canine ribosome, a small portion of L9 is visible on the large-subunit surface (8), which is presumably the N terminus at the...
subunit boundary. Use of trypsin digestion to remove ribosomal proteins located on the surface of the large ribosome subunit failed to remove L9, indicating that it is positioned more internally on the 60S subunit than other ribosomal proteins (14).

Here, we report the unexpected finding that L9 interacts with the MMTV Gag protein in cells infected with MMTV(C3H) virus, a highly tumorigenic strain. Our data indicate that MMTV Gag and L9 interact in the nucleolus, a subnuclear body involved in ribosome biogenesis, cell cycle control, DNA damage responses, and the p53 regulatory feedback loop (reviewed in references 15 and 16). Interestingly, small interfering RNA (siRNA)-mediated knockdown of L9 reduced L9 expression in the nucleolus, and the MMTV yield was also reduced without affecting steady-state levels of the Gag protein. Together, these data suggest that MMTV Gag interacts with extraribosomal L9 during the process of virus assembly.

MATERIALS AND METHODS

Plasmids. pRPL4-GFP and pRPS6-GFP (17) (kind gifts from Tim Kruger, University of Würzburg, Würzburg, Germany), pRSV-Gag-GFP (18), and pGFP-Rem (5) were previously described. pFlagRPL9-GFP (19), a kind gift of Mark Olson (University of Mississippi Medical Center), was modified using PCR cloning to exchange cyan fluorescent protein (CFP) for green fluorescent protein (GFP). pRPL9-FLAG was cloned by amplification of murine rpL9 from total cellular RNA from NMuMG cells using PCR (NCBI NM_011292) and inserted into the BglII site of the pCMV-FLAG-MAT-2 vector (Sigma). pRPL9-mCherry was created by inserting the RPL9 sequence from pRPL9-FLAG into pmCherry-N2, which was made by replacing GFP in pEGFP-N2 (Clontech) with mCherry from pKSet8.mCherry, a kind gift of Roger Tsien (University of California, San Diego) (20). pRPL9-GFP was made by amplification of RPL9 from pRPL9-FLAG and inserted into the HindIII/Sall sites of pEGFP-N2. pMMTV.Gag(C3H)-GFP was created by PCR amplification of MMTV(C3H) gag sequence (6) from plasmid pRFPC-Bait-C used in the yeast two-hybrid assay described below (nucleotide sequence accession number AF228523 for MMTV(C3H) provirus) and insertion into pEGFP-N2 using BamHI-HindIII; 21 nucleotides of the 5' untranslated region (UTR) upstream of Gag were included in the construct. Similarly, pMMTV.Gag(C3H)-mCherry was made by PCR amplification of C3H Gag and insertion into mCherry-N2 using the HindIII/Apal sites. GFP-tagged MMTV(C3H) Gag truncation proteins MA-CA (1 to 1485) and CA (805 to 1485) were PCR amplified at the indicated nucleotides of gag and ligated into the BamHI-HindIII restriction sites of pEGFP-N2. The NC coding region of gag (1486 to 1767) was PCR amplified, digested with BglII, treated with Klenow, digested with BamHI, and ligated into pEGFP.N2 to produce pNC-GFP. pYRPL9B-mCherry was made by amplification of rpl9 isoform B sequences (21) from plasmid pRFPC-Bait-C used in the yeast two-hybrid assay described below and transferring it into SalI-ApaI of pMMTVGag(C3H)-mCherry. The sequence encoding the nucleolar localization signal (NoLS) of the Gag protein in cells infected with MMTV(C3H) virus, a highly tumorigenic strain. Our data indicate that MMTV Gag and L9 interact in the nucleolus, a subnuclear body involved in ribosome biogenesis, cell cycle control, DNA damage responses, and the p53 regulatory feedback loop (reviewed in references 15 and 16). Interestingly, small interfering RNA (siRNA)-mediated knockdown of L9 reduced L9 expression in the nucleolus, and the MMTV yield was also reduced without affecting steady-state levels of the Gag protein. Together, these data suggest that MMTV Gag interacts with extraribosomal L9 during the process of virus assembly.
Coimmunoprecipitation. MMTV-infected NMuMG(C3H) cells were harvested in PBS, incubated in lysis buffer (50 mM Tris, pH 7.05, 1% Triton X-100, 0.5% deoxycholic acid, 150 mM NaCl) for 15 min on ice, and spun for 30 min in a 4°C microcentrifuge at maximum speed to pellet debris. The protein concentration of the cleared lysate was determined by Bradford assay. Protein A Sepharose beads (Invitrogen) were washed twice in coimmunoprecipitation (coIP) buffer (50 mM Tris, pH 8.0, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 150 mM NaCl) and incubated with one of the following antibodies in coIP buffer for 1 h: goat anti-CA (a kind gift from Susan Ross, University of Pennsylvania), goat anti-lamin A/C (Santa Cruz), rabbit anti-RPL9 (AbCam), or rabbit anti-lamin A/C (Santa Cruz). The antibody-bound beads were added to 1.5 mg of total cellular protein per coIP, and the reaction mixtures were incubated twice in coimmunoprecipitation buffer (125 mM Tris-HCL, pH 6.8, 20% glycerol, 0.5% bromophenol blue, 4% SDS, and 10% -mercaptoethanol) prior to analysis by SDS-PAGE and Western blotting.

L9 siRNA-mediated knockdown and virus yield assays. Cells were seeded in 35-mm dishes at a density of 0.25 × 10^6 cells/dish. Twenty-four hours after seeding, the cells were transfected with L9 Smartpool siRNA (Dharmacon; M-042220-01-0005) or scrambled control siRNA (Sigma) for 15 h using Lipofectamine 2000 according to the manufacturer’s instructions. The cells were washed once in standard buffer (100 mM NaCl, 1.0 mM Tris, 1 mM EDTA, pH 7.2) and allowed to recover in primary growth medium for 9 h. The cells were then transfected a second time with L9 or control siRNA for 15 h. After the second transfection, the cells were washed three times in standard buffer, and virus was collected for 3 h in primary growth medium. The medium was removed from the cells and cleared by centrifugation at 2,000 × g at 4°C, layered onto a 25% sucrose cushion, and pelleted by centrifugation at 126,000 × g at 4°C. The pelleted viral particles were resuspended in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). The cells were washed in standard buffer and lysed in RIPA buffer. The protein concentration of cell lysates was determined by Bradford assay, and normalized amounts were loaded onto an SDS-PAGE gel.

Virus yield and L9 expression were determined by Western blotting using the following antibody combinations: mouse anti-MMTV CA with goat anti-mouse-horseradish peroxidase (HRP) (Sigma), rabbit anti-RPL9 (AbCam) with goat anti-rabbit-HRP (Sigma), or goat anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (GeneScript) with rabbit anti-goat-HRP (Sigma). Blots were developed using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) and imaged using an EC3 Chemi HR 410 imaging system (UVG, Upland, CA). The virus yield was calculated as the amount of viral protein detected in the sucrose-purified medium divided by the sum of viral proteins in the lysate plus medium [medium/(lysate + medium)]. L9 expression was normalized to GAPDH loading controls to calculate the relative knockdown of expression. Virus yield assays were performed a minimum of seven times for each condition. The mean, standard error of the mean, and P values were calculated using GraphPad Prism 5 (GraphPad Software, Inc.).

RESULTS

We previously described a group of replication-competent exogenous MMTV strains that differ in their propensity to cause tumors in mice (6, 34). Compared to the highly tumorigenic strain MMTV(C3H), a tumor-attenuated virus was identified that arose from a recombination event between MMTV(C3H) and an endogenous retrovirus, Mtv1, to create the MMTV(HP/Mtv1) strain (6, 34). The difference in tumorigenicity between the MMTV(C3H) and MMTV(HP/Mtv1) strains of exogenous MMTV maps to a 253-amino-acid region within the CA and NC regions of Gag (6).

Yeast two-hybrid screen. To identify cellular proteins that might play a role in MMTV Gag-mediated tumorigenesis, a yeast two-hybrid screen was used to identify host proteins that differentially interacted with MMTV(C3H) or MMTV(HP/Mtv1) Gag. As bait, we used a series of hybrid Gag proteins that contained replacements of the tumor-attenuated MMTV(HP/Mtv1) gag sequence with corresponding regions from the highly tumorigenic MMTV(C3H) gag gene (Fig. 1A) (34). cDNA libraries made from the mammary glands of C3H/HeN and BALB/c mice were used as prey. L9 was the only protein found to interact more strongly with Gag proteins derived from viruses with higher tumor incidence (virus A [virA] and virus C [virC]) than with tumor-attenuated viruses (HP/Mtv1 and virus B [virB]) (Fig. 1B, right). Quantitation of the Gag-L9 interactions revealed the highest degree of binding between virA Gag and L9, while virC Gag demonstrated intermediate binding. The virB and Mtv1 Gag proteins produced the weakest interactions (Fig. 1C). The observation that the strength of the interaction between Gag and L9 in yeast cells was correlated with the tumorigenicity of the virus led us to investigate whether L9 and MMTV Gag interacted in cultured mouse mammary (NMuMG) cells. To this end, we first examined the subcellular localizations of L9 and the Gag protein of MMTV(C3H), which is identical to that of gagvirA used in the yeast two-hybrid analysis (Fig. 1) (34).

Cell line-specific localization of MMTV Gag to nucleoli. In mouse mammary cells stably transfected with an MMTV(C3H) proviral expression vector (NMuMG-C3H), Gag formed discrete cytoplasmic foci with exclusion of the nucleus when imaged using deconvolution microscopy (Fig. 2A, top row, yellow arrow). In contrast, we were surprised to find a population of Gag that localized to nucleoli in NMuMG cells infected by coculture with MMTV(C3H)-infected MM5MT cells isolated from mouse tumors (NMuMG-MM5MT) cells also revealed Gag within nucleoli (Fig. 2B, white arrows), indicating that Gag localized to nucleoli in cells newly infected via cell-to-cell spread.

L9 is a component of the large ribosomal subunit (35, 36). In NMuMG cells, endogenous L9 was localized to the cytoplasm and to nucleoli, the site of preribosome assembly and where L9 colocalized with fibrillarin-GFP (Fig. 2C, top row, yellow arrow). We also noticed that the L9 antibody stained mitotic spindles in di-
viding cells, a finding that had not been reported previously (Fig. 2C, bottom row). Based on the observation that a subpopulation of Gag was localized in the nucleoli of NMuMG-MM5MT cells, we tested whether L9 overexpression would alter Gag localization in NMuMG-C3H cells. To differentiate transfected L9 from the endogenous protein, we created epitope (FLAG)- or fluorophore (GFP or mCherry)-tagged L9 constructs. As expected, L9-mCherry colocalized with fibrillarin-CFP in nucleoli of NMuMG cells (Fig. 2D, top row). Interestingly, when L9 was overexpressed in NMuMG-C3H cells, Gag (detected using an α-G9 antibody) accumulated in nucleoli in 78% of the cells (n = 23) overexpressing L9-mCherry (Fig. 2D, bottom row). Co-localization analysis of the cells where Gag was relocalized by L9-mCherry expression revealed that, on average, 18% ± 4% of the Gag signal colocalized with L9 (M1) and 55% ± 5% of the L9 signal colocalized with Gag (M2) (n = 18) (Fig. 2D, bottom row), suggesting that L9 overexpression induced Gag to accumulate in nucleoli in NMuMG-C3H cells.

Finding a difference in the localization of Gag in NMuMG-C3H versus NMuMG-MM5MT cells was unexpected because the Gag proteins expressed in these cells are identical, as confirmed by isolation of total cellular RNA from both cell lines and sequencing of the \( \text{gag} \) gene using reverse transcription (RT)-PCR (data not shown). Thus, the variation in Gag localization may be due to a difference in a cellular factor or a viral component other than Gag. Although it was feasible that NMuMG-MM5MT cells might exp-
press higher basal levels of L9 than NMuMG-C3H cells as an explanation for the nucleolar localization of Gag in NMuMG-MM5MT cells, we did not observe a significant difference in L9 levels by Western blotting (data not shown). Therefore, either the Western analysis was insufficiently sensitive to detect differences in L9 levels or a host factor in addition to L9 contributes to Gag nucleolar localization.

To test whether Gag would relocalize to nucleoli with L9 overexpression in the absence of other viral factors, we created GFP- and mCherry-tagged MMTV(C3H) Gag constructs (Gag-GFP and Gag-mCherry, respectively) (Fig. 3A). Gag-GFP and Gag-mCherry formed small cytoplasmic foci in uninfected NMuMG cells, which were similar in size and distribution to Gag foci observed in NMuMG-C3H cells (Fig. 2A). When fluorophore-tagged Gag and L9 constructs were cotransfected into uninfected NMuMG cells, Gag accumulated in nucleoli in 66% of cotransfected cells (n = 33) (Fig. 3B, yellow arrow). Among cells where Gag was relocalized by L9 expression, 16% ± 3% of Gag colocalized with L9 and 71% ± 3% of L9 colocalized with Gag (n = 22). Similar results were observed when Gag-GFP was cotransfected with FLAG-tagged L9 (L9-FLAG) (Fig. 3B, yellow arrow). Thus, the subcellular distribution of MMTV Gag was altered by L9 expression in the absence of any additional viral proteins or the viral genome. Because of the higher degree of binding in the two-hybrid assay between L9 and MMTV(C3H) Gag compared to MMTV(HP/Mtv1), we tested whether L9 overexpression would change the localization of Gag derived from Mtv1. In fact, L9 overexpression also induced localization of a subpopulation of Mtv1 Gag to nucleoli (data not shown), indicating that the difference in binding affinity observed in the quantitative yeast two-hybrid assay was not apparent in NMuMG cells, which are derived from Namru mice (37–39). It is possible that Namru mice, like BALB/cJ mice, do not exhibit differences in the rates of tumor formation with variants of MMTV tested by Swanson et al. (34) (Fig. 1). Therefore, future experiments will need to be performed in mammary cells derived from BALB/cJ mice to address whether L9 interacts preferentially with MMTV(C3H) Gag compared with Mtv1 Gag. In this report, we instead focused on characterizing the interaction of L9 with MMTV(C3H) Gag to learn more about its relevance to virus assembly.

To determine whether L9-induced relocalization of Gag was unique to MMTV, the GFP-tagged RSV Gag protein was cotransfected with murine RPL9-mCherry in quail fibroblasts. Overexpression of RPL9-mCherry did not affect the subcellular distribution of RSV Gag (Fig. 3C), suggesting that the interaction between Gag and L9 was limited to MMTV. To examine whether the relocalization of MMTV Gag could be induced by overexpression of Cys5-conjugated secondary antibody (red) and stained using DAPI (blue) to show nuclei. The yellow arrows point to L9 staining within nucleoli. (Bottom) Mitotic spindle stained with anti-L9 antibody. (D) (Top) Representative NMuMG cell coexpressing fibrillarin-CFP (false colored green) and RPL9-mCherry (red) with colocalization (yellow) shown in the overlay image. The dotted line indicates the outline of the cell. (Bottom) Expression of RPL9-mCherry (red) in NMuMG-C3H cells immunostained for Gag (green). M1 and M2, colocalization coefficients were measured by Mander’s analysis using ImageJ JACoP as described in Materials and Methods and are shown for the representative cell outlined with a white dotted line. M1 indicates the percent age of fluorescence in the green channel that is colocalized with signal in the red channel, and M2 is the converse.
other ribosomal proteins, RPS6 and RPL4, each fused to GFP, were expressed in NMuMG-C3H cells. Both RPS6-GFP and RPL4-GFP localized to nucleoli, similarly to L9; however, MMTV Gag distribution was unaffected by S6 or L4 overexpression (Fig. 3D). Together, these data indicate that MMTV Gag specifically interacted with L9 and that this interaction may not be conserved among other retroviruses.

**Gag interacts with L9 in nucleoli.** To this point, our data demonstrated that although Gag was not visualized in nucleoli of NMuMG-C3H cells under steady-state conditions, overexpression of L9 induced a subpopulation of Gag to accumulate within nucleoli. To assess whether the association of Gag and L9 could be detected biochemically, we conducted coIP experiments using anti-CA or anti-RPL9 antibodies to isolate protein complexes from NMuMG-C3H cells. MMTV Gag was coimmunoprecipitated using an anti-RPL9 antibody, but not a nonspecific isotype control antibody (anti-lamin A/C IgG) (Fig. 4A, top). Similarly, a reciprocal experiment demonstrated that L9 was pulled down with an anti-CA antibody, but not with the nonspecific control antibody (anti-lamin B IgG) (Fig. 4A, bottom). It is likely that the L9 band ran higher in the immunoprecipitation lane than the unbound lanes due to differences in the salt concentrations of the samples, which may noticeably alter the electrophoretic properties of the 21-kDa L9 protein. Furthermore, Gag also communoprecipitated with L9 from concentrated preparations of nucleoli (data not shown). Together, these data demonstrate that Gag and L9 form an intracellular complex in MMTV-infected mammary cells.

Next, we used AP-FRET to determine whether Gag and L9 were interacting directly within nucleoli. As we observed previously, expression of RPL9-mCherry resulted in Gag accumulation in nucleoli of MMTV-infected NMuMG-C3H cells, which were immunostained for Gag using anti-CA (α-CA) antibody and FITC-labeled secondary antibody (Fig. 4B). Photobleaching of nucleolar RPL9-mCherry with a 543-nm laser resulted in decreased mCherry fluorescence (Fig. 4B, compare top row, pre- and postbleach images, white arrows) with a concomitant increase in FITC fluorescence (compare bottom row, pre- and postbleach images, yellow arrows), indicating a significant intermolecular transfer of resonance energy between the FITC and mCherry chromophores, which was clearly visible in the images. The mean AP-FRET efficiency between FITC-stained Gag and RPL9-mCherry was 67.9% ± 13% (Fig. 4D). Because FRET occurs only when fluorophores are in very close proximity (i.e., <50 Å for enhanced GFP [EGFP] and mCherry) (40), these data suggest that Gag and L9 bind to each other within nucleoli. As a control for the FRET experiment, RPL9-mCherry was coexpressed with MMTV GFP-Rem, which also localizes to nucleoli (5) (Fig. 4C). In this case, RPL9-mCherry was bleached (compare top row, pre- and postbleach images, white arrows), but there was no visible increase in intensity of the GFP-Rem fluorescence (Fig. 4C, compare bottom row, pre- and postbleach images, yellow arrows), and the AP-FRET efficiency between the two proteins was only 7.9% ± 5.7% (Fig. 4D). As additional controls, mCherry alone did not demonstrate efficient FRET with FITC-labeled Gag in NMuMG-C3H cells (10.7% ± 6.5%), and the background level of AP-FRET between mCherry and GFP was 3.9% ± 2.1% (Fig. 4D), indicating that the high level of AP-FRET between FITC-labeled Gag and RPL9-mCherry was specific for Gag and L9. Taken together, the results of these experiments indicate that Gag and L9 form a com-

![FIG 3](https://example.com/fig3.png)

**FIG 3** MMTV Gag specifically interacts with RPL9. (A) (Top) Diagram of MMTV Gag-GFP showing the cleavage sites of Gag (MA, pp21, P3, p8, n, CA, and NC). (Bottom) Expression of fluorophore-tagged MMTV Gag in uninfected NMuMG cells imaged using confocal microscopy. (B) Coexpression of MMTV Gag-mCherry with RPL9-GFP (top row) and MMTV Gag-GFP with RPL9-FLAG (bottom row) in uninfected NMuMG cells. Mander’s coefficient for colocalization is shown for the representative cell outlined with a white dotted line, as described in the legend to Fig. 2. (C) RSV Gag-GFP was coexpressed with RPL9-mCherry in QT6 cells. Mander’s coefficient for colocalization is shown for the representative cell outlined with a white dotted line. (D) RPL4-GFP or RPS6-GFP was expressed in MMTV-infected NMuMG-C3H cells immunostained for Gag (green). Mander’s coefficient for colocalization was measured on the cell outlined with a white dotted line.
plex in nucleoli, likely through a direct protein-protein interaction.

The CA domain is the major determinant of Gag-L9 interaction. We next sought to identify the region of Gag that interacts with L9. Single domains of MMTV Gag or Gag truncation mutants tagged with GFP (Fig. 5A) were expressed in uninfected NMuMG cells with or without RPL9-mCherry. When expressed alone, MMTV NC-GFP was nucleolar, as determined by the identification of nucleoli in the difference interference contrast (DIC) microscopy images (Fig. 5B, yellow arrow). In contrast, when NC was deleted from Gag (MA-CA.GFP), the truncated protein was predominantly cytoplasmic, with faint nuclear fluorescence that excluded nucleoli (Fig. 5C, top). However, when coexpressed with RPL9-mCherry, MA-CA.GFP localized exclusively to nucleoli (Fig. 5D, top row). Quantitation of the representative images in the top row of Fig. 5D using Mander’s analysis revealed that 100% of the MA-CA.GFP protein colocalized with L9 (M1), and reciprocally, 86% of the L9 signal overlapped with MA-CA.GFP (M2).

To determine whether the CA region was sufficient to mediate the interaction between Gag and L9, the subcellular distribution of CA-GFP was tested in the absence or presence of L9 overexpression. CA-GFP was diffuse throughout the cell, with nucleolar exclusion when expressed alone (Fig. 5C, middle). However, coexpression with RPL9-mCherry resulted in a striking relocalization of CA-GFP to nucleoli and complete colocalization with L9 (Mander’s analysis; M1/100%; M2/93%) (Fig. 5D, middle row). In contrast, expression of RPL9-mCherry had no effect on the distribution of GFP alone (Fig. 5C, bottom). These results indicate that the major determinant of the Gag-L9 interaction maps to the CA region, although other regions may play contributing roles.

Gag-L9 interaction is conserved in lower eukaryotes. To gain insight into the region(s) of L9 that interacts with MMTV Gag, we compared the amino acid sequence of murine L9 to those of several other eukaryotic species (Table 1). L9 was highly conserved among vertebrates, with the murine amino acid sequence bearing high homology and identity to human (99% homology; 98% identity), rat (99% homology; 98% identity), chicken (98% homology; 94% identity), and zebrafish (95% homology; 89% identity) sequences. As expected, there was less sequence conservation between murine and D. melanogaster L9 (80% homology; 64% identity). Interestingly, two isoforms of L9 have been reported in yeast, differing by only 3 amino acids (41, 42). Murine L9 was 69% homologous and 49% identical to yeast L9A and 69% homologous and 50% identical to yeast L9B (Fig. 6A). To test whether yeast L9 would also relocalize MMTV Gag, we expressed bleaching the nucleus with a 543-nm laser to remove most of the RPL9-mCherry fluorescent signal (white arrows). MMTV Gag was detected by immunofluorescence using anti-CA primary antibody and FITC-labeled secondary antibody, with prebleach and postbleach images shown. The yellow arrows point to the dramatic increase in fluorescence signal of Gag-FITC in nucleoli due to dequenching following bleaching of RPL9-mCherry. (C) GFP-Rem and RPL9-mCherry coexpressed in NMuMG cells. Confocal microscopy images were acquired and are shown on the left. Postbleach images were obtained after bleaching the nucleus with a 543-nm laser to remove most of the RPL9-mCherry fluorescent signal (white arrows). MMTV Gag was detected by immunofluorescence using anti-CA primary antibody and FITC-labeled secondary antibody, with prebleach and postbleach images shown. The yellow arrows point to the dramatic increase in fluorescence signal of Gag-FITC in nucleoli due to dequenching following bleaching of RPL9-mCherry. (D) Comparison of AP-FRET efficiency values obtained from the following protein pairs: RPL9-mCherry and viral CA/Gag (donor) and MMTV Gag-FITC (acceptor) in MMTV(C3H)-infected NMuMG cells. Confocal images represent raw data used for FRET analysis. Prebleach images were acquired and are shown on the left.
mCherry-tagged yeast L9B in NMuMG-C3H cells. Interestingly, yeast L9B localized to nucleoli in murine cells, suggesting that its nucleolar localization signal (NoLS) remained functional, and it induced Gag to accumulate in nucleoli (Fig. 6B). This result indicates that the region of L9 that interacts with MMTV Gag resides in the portion of the protein that is conserved between murine and yeast L9.

Gag interacts with the C-terminal region of L9. Sequence comparison between the mouse and yeast L9 proteins revealed the highest level of conservation within the C-terminal region of L9 (Fig. 6A; the red vertical line denotes the junction of N- and C-terminal domains). To determine whether the C-terminal region of L9 was sufficient to interact with Gag, we coexpressed mCherry-tagged N- or C-terminal fragments of L9 (Fig. 7A) with Gag-GFP in NMuMG cells. The N-terminal fragment of L9 (RPL9.NT-mCh) localized to nucleoli of NMuMG cells. However, Gag-GFP did not accumulate within nucleoli of cells expressing the N-terminal fragment (Fig. 7B, top row). In contrast, the C-terminal fragment of L9 (RPL9.CT-mCh) accumulated in small cytoplasmic foci that partially colocalized with Gag-GFP (Manders analysis; $M_1 = 36\%$; $M_2 = 25\%$) (Fig. 7B, bottom row). No colocalization was seen in MMTV-infected NMuMG-C3H cells between Gag and RPL9.NT-mCh (Fig. 7C, top row), and again, partial colocalization was observed between the C-terminal region of L9
and Gag in cells expressing RPL9 CT-mCh (M₁ = 15%; M₂ = 18%) (Fig. 7C, bottom row). These data suggest that the NoLS for L9 is in the N-terminal half of the protein, whereas the C-terminal portion may contain the Gag interaction domain.

To test whether the C terminus of L9 was sufficient to relocalize Gag to nucleoli, we fused a heterologous NoLS from the HIV-1 Rev protein onto the L9 C-terminal domain in an attempt to re-store proper localization of L9 (Fig. 7D). First, we demonstrated that the Rev NoLS would drive mCherry into the nucleolus (RevNoLS-mCherry) (Fig. 7E, top row, middle image). However, this nucleolar protein was not capable of relocalizing Gag-GFP (Fig. 7E, top row, left image). In contrast, when Gag-GFP was coexpressed with the C-terminal fragment of L9 fused to the Rev NoLS (RPL9 CT-Rev-mCherry), Gag-GFP was relocalized to nucleoli and partially colocalized with the L9 chimeric protein in nucleoli (Fig. 7E, bottom row, yellow arrow) (M₁ = 4%; M₂ = 92%). Thus, the C-terminal domain of L9 was sufficient for relocalization of Gag, provided that L9 contains a functional NoLS. Interestingly, deleting the C-terminal 26 amino acids, which contain the highest degree of conservation between mouse and yeast L9B (Fig. 6A), from L9 did not disrupt the Gag-L9 interaction, indicating that this sequence is not required for Gag to interact with L9 (data not shown).

Knockdown of L9 expression interferes with MMTV production. Finding that L9 and Gag interacted in the nucleolus raised the possibility that L9 could play a role in virus production. To test this idea, we used a pool of three L9-specific siRNAs to reduce L9 expression by approximately 35% compared to scrambled control siRNA (P < 0.05) (Fig. 8A), relative to GAPDH, the internal control. The modest L9 knockdown likely results from long-lived L9 present in ribosomes, which are turned over as a functional unit with a half-life of 96 to 120 h (43, 44), and therefore would be largely unaffected by the transient knockdown. Indeed, when we examined the L9 distribution in siRNA-treated cells by immunofluorescence, we observed no difference in the distribution or the amount of cytoplasmic L9 (Fig. 8B). However, in L9-specific siRNA-transfected cells, L9 was not detected in nucleoli, indicating that the knockdown primarily affected the extraribosomal pool of L9 prior to its incorporation into ribosomes. Furthermore, the siRNA-treated cells maintained normal morphology, suggesting that the L9 knockdown was not overly toxic, and the cytoplasmic distribution of Gag was not obviously altered (Fig. 8B). Because L9 is essential for ribosomal function, we next tested whether this degree of L9 knockdown affected steady-state levels of Gag. We found that L9 knockdown did not result in a significant decrease in Gag expression relative to GAPDH loading controls (Fig. 8C). However, the virus yield from cells treated with L9 siRNA was reduced by ~50% relative to the scrambled siRNA control (P < 0.005) (Fig. 8D) when normalized to intracellular Gag levels. This finding suggests that the Gag-L9 interaction is intrinsic to the virus assembly and budding pathway, although we cannot rule out the possibility that the defect in virus production is due to an indirect effect of the L9 siRNA treatment.

DISCUSSION
Retrovirus-cell interactions that contribute to the processes of virus assembly and budding remain incompletely understood. In particular, the functions of host factors that engage retroviral Gag proteins far from the site of virus budding are difficult to unravel. In this work, we report the novel finding that L9, a protein that resides in the nucleolus prior to its incorporation into ribosomes, interacts with the MMTV Gag protein and influences virus assembly. This report is the first to find an interaction between Gag and...
We propose that virus assembly may be modulated by this interaction.

Why is the MMTV Gag protein present in the nucleolus in association with L9, a constituent of the large preribosome complex? These data raise the intriguing possibility that L9 has an extraribosomal function, possibly one that contributes to Gag-mediated virus assembly. Recently, overexpression of ribosomal protein L4 was found to increase readthrough of the murine leukemia virus (MLV) pseudoknot, which is required to produce the MLV Gag-Pol fusion protein (45). This imbalance in the Gag/Gag-Pol ratio led to an assembly defect, possibly due to steric constraints imposed by excess Gag-Pol on virus particle assembly. MMTV uses ribosomal frameshifting rather than readthrough to produce Gag-Pro and Gag-Pro-Pol fusion proteins (46). In our experiments, we did not detect a change in the Gag/Gag-Pol ratio by Western blotting following L9 knockdown, suggesting that the reduction in particle production we observed was unlikely to be due to a change in the efficiency of frameshifting.

It is not evident from our data whether the effect of L9 on virus assembly is directly related to an extraribosomal activity of L9 or whether another host factor is recruited into the Gag-L9 complex. The influence of L9 appears to occur early in the assembly pathway, as we have no evidence that L9 colocalizes with Gag at cytoplasmic sites of capsid assembly, nor have we detected L9 in virus particles (data not shown). Moreover, because Gag and L9 associated in the nucleolus, other nucleolar proteins or RNAs may contribute to virus assembly. Candidates include nucleolin and B23, which modulate HIV-1 assembly (47–52), and small nuclear RNAs, products of Pol III transcription at the nucleolar periphery, which are selectively incorporated in retrovirus particles (53–57).

**FIG 7** Mapping the Gag interaction domain within L9. (A) Schematic of the N-terminal and C-terminal regions of RPL9 fused to mCherry. RPL9.NT consists of RPL9 amino acids 1 to 85, and RPL9.CT consists of amino acids 86 to 192. (B) Confocal microscopy of NMuMG cells cotransfected with MMTV(C3H) Gag-GFP and mCherry-fused RPL9 N- or C-terminal domains. (C) Confocal microscopy of MMTV(C3H)-infected NMuMG cells transfected with the N- or C-terminal RPL9-mCherry construct. Gag was detected with anti-CA primary antibody and FITC-conjugated secondary antibody. (D) Schematic diagram of the HIV-1 Rev NoLS fused to the N terminus of mCherry and inserted into the RPL9.CT-mCherry construct between the RPL9 and mCherry sequences. (E) Confocal images of Gag-GFP coexpressed with Rev NoLS-containing constructs. For each of the overlay images, M1 and M2 colocalization values for single cells or those outlined with white dots were calculated using ImageJ JACoP.
Figure 8: Effect of L9 depletion on MMTV virus yield. MMTV-infected NMuMG-C3H cells were treated with L9-specific or scrambled control siRNAs. (A) Effect of L9 siRNA treatment on L9 expression relative to GAPDH loading controls, with the error bars representing the mean ± standard error of the mean (n = 7). A two-tailed Student’s t test was performed to compare the treatment groups; *, P < 0.05. A representative Western blot is shown on the right of the graph, demonstrating selective reduction in L9 expression. (B) Cells treated with L9-specific or control siRNAs were immunostained for L9 and imaged using fluorescence confocal microscopy. The yellow arrows in the scrambled siRNA images point to L9 immunofluorescence in nucleoli, which is absent from the L9-siRNA-treated cells below. (C and D) The samples from panel A were used to evaluate steady-state Gag levels (C) and virus yield (D) relative to scrambled siRNA treatment and normalized for GAPDH expression levels. The virus yield was calculated as the amount of Gag in the sucrose-purified media divided by the sum of Gag in the lysates and media. Representative Western blots from each experiment are shown on the right of the graphs. The graphs show means ± standard errors of the mean; **, P < 0.005.

Future experiments will explore whether modulating L9 expression levels alters MMTV infectivity, incorporation of viral genomic RNA, or enrichment of Pol III-transcribed cellular RNAs into virions.

Localization of MMTV Gag to the nucleus or nucleolus has not been reported previously, although other retroviral Gag proteins do traffic transiently through the nucleus (references 58–65; reviewed in reference 66). For RSV, genetic and biochemical evidence suggests that genomic RNA packaging is linked to nuclear trafficking of Gag (33, 65, 67), but whether this role is conserved for other retroviruses is not known. What is the mechanism of MMTV Gag nucleolar localization? Finding that the MMTV NC protein contains nucleolar localization activity suggests that it may contain the Gag NoLS. In addition, our data clearly showed that increasing the intracellular level of L9 induced the accumulation of Gag in nucleoli through an interaction with the CA region. Thus, it is feasible that the degree of MMTV Gag nucleolar localization varies due to cell type differences in L9 or other factors. It is also possible that Gag-L9 interactions are initiated in the cytoplasm and Gag piggybacks into the nucleolus with L9. Further experiments will be needed to differentiate between these possibilities.

Interestingly, many ribosomal proteins have well-established extraribosomal functions mediated by their RNA binding properties and protein–protein interaction domains. For example, several ribosomal proteins autoregulate their synthesis by altering splicing or translation of their own mRNA (reviewed in reference 68). Furthermore, L26 binds to the 5′ UTR of p53 mRNA, stimulating p53 translation (69), and extraribosomal L11 modulates levels of c-myc by recruiting microRNA (miRNA) and a component of the RNA-induced silencing complex to the 3′ UTR of the c-myc gene (70) in response to nucleolar stress. Other ribosomal proteins engage in protein–protein interactions that modulate cellular functions, including those that serve as sentinels to identify states of cellular stress triggered by ribosomal dysfunction. Specifically, ribosomal proteins L5, L11, L23, and S7 activate p53 by binding to MDM2, resulting in cell cycle arrest or apoptosis (71–74). L23 sequesters nucleophosmin in the nucleolus, preventing it from interacting with a c-myc antagonist, leading to stimulated cell growth (75). Ribosomal protein S3 can be induced to undergo nuclear import, where it binds to NF-κB to stimulate gene-specific transcription (76). As further evidence of their critical extraribosomal functions, mutations in ribosomal proteins are associated with the human diseases Diamond-Blackfan anemia and 5q syndrome, and dysregulated ribosomal protein expression occurs in cancers, such as hepatocellular, colorectal, and prostate cancer and melanoma, sarcoma, and lymphoma (77, 78). L9 and other ribosomal proteins act as tumor suppressors, consistent with observations that mutations in genes encoding ribosomal proteins lead to malignant diseases (12, 79–82).
be suggested that they are prime targets of viruses, which commonly hijack host machinery to facilitate their own replication (68). Accordingly, it is not surprising that diverse families of viruses have been reported to interact with ribosomal proteins. During hepatitis C virus (HCV) infection, S5 and S9 interact with the internal ribosome entry site (IRES) on the HCV RNA to position the IRES on the 4OS ribosomal subunit for optimal translation of viral proteins (83). The noncoding EBER-1 RNA of Epstein-Barr virus (EBV) binds to and relocalizes L22, although the biological relevance of this finding in EBV infection is not known (84, 85). Among retroviruses, infection with RSV or Abelson murine leukemia virus (Ab-MuLV) results in phosphorylation of S6 (86, 87). The role of S6 phosphorylation in retrovirus infection is unclear, but data from cells transformed with simian virus 40 (SV40) suggest that S6 phosphorylation may promote cell growth and oncogenic transformation (88). Recently, it was shown that L4 modulates Moloney murine leukemia virus translational readthrough, which is required for maintenance of the proper Gag/Gag-Pol ratio (45). Up to now, no other retroviral proteins have been reported to interact with ribosomal proteins either within ribosomal subunits or in an extraribosomal setting.

Interestingly, two other MMTV-encoded proteins, the Rem nuclear export factor (5) and the signal peptide of Rem and Env, known as p14 (89), independently localize to nucleoli in infected cells. Therefore, it is plausible that Gag, Rem, and/or p14 encounter one another within nucleoli. The p14 protein was recently found to regulate transcription levels of ribosomal protein L5, which interferes with MDM2-mediated degradation of p53 (7, 90). In addition, the MMTV Env protein transforms mammary cells in culture (7), and enhanced tumorigenicity in vivo maps to the gag gene (6). Thus, the mechanism by which MMTV induces tumors in mice appears to be more complex than previously realized. The newly appreciated role of the nucleolus and the ribosomal proteins that interact with MMTV proteins opens the door to advancing our understanding of how viruses intersect with host regulatory pathways to replicate and cause disease.

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Appendix B

Alterations in the MA and NC Domains Modulate Phosphoinositide-Dependent Plasma Membrane Localization of the Rous Sarcoma Virus Gag Protein

Darrin V. Bann*, Shorena Nadaraia-Hoke*, Timothy L. Lochmann, Nicole Gudleski-O'Regan, and Leslie J. Parent
*Authors contributed equally

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Alterations in the MA and NC Domains Modulate Phosphoinositide-Dependent Plasma Membrane Localization of the Rous Sarcoma Virus Gag Protein

Shorenâ€œ Nadaraia-Hoke,a Darrin V. Bann,a Timothy L. Lochmann,a Nicole Gudlesi-O’Regana, Leslie J. Parentab,c
Departments of Medicinea and Microbiology and Immunologyb and the MVPhD Program, Penn State College of Medicine, Hershey, Pennsylvania, USA

Retroviral Gag proteins direct vesicle assembly from the plasma membrane (PM). Phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2] plays a role in PM targeting of several retroviral Gag proteins. Here we report that depletion of intracellular PI(4,5)P2 and phosphatidylinositol-(3,4,5)-triphosphate [PI(3,4,5)P3] levels impaired Rous sarcoma virus (RSV) Gag PM localization. Gag mutants deficient in nuclear trafficking were less sensitive to reduction of intracellular PI(4,5)P2 and PI(3,4,5)P3, suggesting a possible connection between Gag nuclear trafficking and phosphoinositide-dependent PM targeting.

The retroviral G polypeptide orchestrates the assembly of virus particles, which are released from the plasma membrane (PM) of infected cells. The major domains of G includes matrix (MA), capsid (CA), and nucleocapsid (NC), with MA containing the myristoylation signal (I–9). Studies to determine how MA directs membrane targeting of G revealed that interactions with phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2] and other negatively charged lipid molecules are required for proper localization of several retroviral Gag proteins, including human immunodeficiency viruses type 1 and type 2 (HIV-1 [10–13] and HIV-2 [12]), murine leukemia virus (MLV) (1, 14), and Mason-Pfizer monkey virus (MPMV) (3, 7). In the case of HIV-1, Gag is directed to the PM by a bipartite signal in MA consisting of N-terminal myristic acid modification and a cluster of basic amino acids (9). Stable association of HIV-1 Gag with the PM is achieved when PI(4,5)P2 is bound by this basic cleft in MA, leading to insertion of the myristoyl chain into the PM (10, 13). Similarly, MLV MA is myristoylated and binds PI(4,5)P2 in the presence of phosphatidylyserine but lacks an obvious PI(4,5)P2 binding cleft (1). The MPMV MA protein must be myristoylated to bind PI(4,5)P2; if it binds with lower affinity than does HIV-1 or HIV-2 MA (3). In contrast, equine infectious anemia virus and human T-lymphotropic virus type 1 are less dependent on HIV-1 on specific binding of PI(4,5)P2 for Gag recruitment to the PM and particle release (16–18).

Rous sarcoma virus (RSV) Gag, which is acetylated (19, 20) but not myristoylated, undergoes nuclear trafficking prior to PM localization owing to nuclear localization signals (NLSs) in the MA and NC domains. Nuclear export is mediated by a nuclear export signal (NES) in p10, an RSV-specific sequence upstream of the CA domain (21–23). The NLS in MA overlaps the membrane-binding domain (MBD), suggesting the need for an ordered sequence of trafficking events from the nucleus to the plasma membrane (8). However, the mechanism by which RSV Gag is targeted to the PM after exit from the nucleus is not well understood.

The RSV Gag MBD spans the first 86 amino acids of MA and contains 11 basic residues that form a basic patch on a surface-exposed region of the protein, similarly to other retroviral MBDs (8, 24, 25). The structural similarity between the RSV MBD and those of other retroviruses suggests that RSV Gag may interact with acidic phospholipids via electrostatic interactions (24, 26). A previous study of RSV Gag membrane targeting reported that PM localization in cells was unaffected by PI(4,5)P2 and phosphatidylinositol-(3,4,5)-triphosphate [PI(3,4,5)P3] depletion, although the authors found that both of these phosphoinositides enhanced Gag binding to liposomes in vitro (27). In contrast, we report that expression of the enzyme inositol polyphosphate 5-phosphatase type IV (SptaseIV), which dephosphorylates levels of PI(4,5)P2 and PI(3,4,5)P3 by dephosphorylation of position 5 (28), significantly reduced RSV Gag PM localization and virus-like particle (VLP) assembly. Interestingly, we found that Gag mutants varied in their sensitivity to SptaseIV activity, and mutants we tested that are impaired in nuclear trafficking were less dependent on PI(4,5)P2 and PI(3,4,5)P3 for PM binding.

To examine the possibility that the wild-type RSV Gag protein might bind to regions of the PM similar to the pleckstrin homology (PH) domain from phospholipase C8, which specifically binds PI(4,5)P2 on the inner leaflet of the PM (17, 29, 30), we examined the membrane localization of RSV Gag compared to that of the isolated PH domain (pPH-mCherry) by PCR amplifying the mCherry sequence from pRSETa-mCherry (a gift from R. Tsien, University of California at San Diego) (31) and exchanging the sequence with green fluorescent protein (GFP) in pGFP-PH/H9254 (a gift from C. Carter, Stony Brook University) (17, 29) using Agel-BglI. The fusion proteins were expressed in QT6 quail fibroblast cells, which are a natural host for RSV. QT6 cells were transfected with 0.5 μg of each plasmid DNA using the calcium phosphate method (32, 33). At 15 h posttransfection, cells were fixed in 2% paraformaldehyde, permeabilized with methanol at −20°C, immunostained (if needed), mounted on slides using SlowFade reagent (Invitrogen), and imaged through the center of the nuclear plane using a Leica SP2 laser-scanning confocal microscope (Leica Microsystems).
RSV Gag-GFP and PH-mCherry both localized to the PM of QT6 cells, with 0.26 ± 0.02 of the PH-mCherry signal overlapping with Gag (M₁) and 0.54 ± 0.04 of the Gag signal overlapping with PH-mCherry (M₂) (n = 10). Consistent with previous reports, epifluorescence of HIV-1 Gag-GFP (34) (a gift from Mari- lyn Resh, Memorial Sloan-Kettering Cancer Center) also overlapped with PH-mCherry (M₁ = 0.29 ± 0.04, M₂ = 0.49 ± 0.03; n = 10) (30) (Fig. 1A and B). Therefore, in QT6 cells, both RSV and HIV-1 Gag proteins were recruited to similar regions of the PM as the P(4,5)₂⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-~ binding protein PH-mCherry.

To examine whether RSV Gag localization was altered by enzymatic depletion of PI(4,5)₂⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~- and PI(3,4,5)₃⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~- (30), a catalytically inactive mutant of SptaseIV (gifts of E. Freed, NCI-Frederick), in QT6 cells and visualized the proteins with rabbit anti-Myc antibody (AbCam) and goat anti-rabbit secondary antibody conjugated to Cy3 (Sigma) or Cy5 (Invitrogen). The subcellular localization of Gag was visually scored as predominantly localized to the PM (M) or cytoplasm (C) in >20 cells transfected on separate days. To avoid bias, researchers were blinded to the identity of the sample at every stage of the experiment (during transfection, image acquisition, and scoring of subcellular localization). Scoring of Gag subcellular localization was performed independently by two investigators, with excellent reproducibility of the results. The statistical significance of changes in Gag localization was determined by Fisher’s exact test (GraphPad Prism).

RSV Gag-GFP was localized to the PM under steady-state conditions in the majority of cells (M = 90%, C = 10%, n = 32)
Phosphoinositide-Dependent Localization of RSV Gag

In contrast, SptaseIV coexpression resulted in a significant reduction in the PM localization of Gag (M = 31%, C = 69%, n = 20; p < 0.0001), whereas SptaseIVΔ1 did not markedly alter Gag localization (M = 70%, C = 30%, n = 32; p > 0.10). To determine whether VLP production was also affected by the reduction in intracellular PI(4,5)P$_2$ and PI(3,4,5)P$_3$ levels, cells expressing Gag-GFP with or without SptaseIV or SptaseIVΔ1 were metabolically labeled with $[^35]$methionine-cysteine. Cell lysates and clarified supernatants were immunoprecipitated using anti-RSV antibody, and radioactive bands were quantified by PhosphorImager analysis as previously described (35–37). Budding efficiency was calculated as the ratio of Gag-GFP in the supernatant to the total Gag-GFP (cell lysate plus supernatant). Expression of SptaseIV reduced Gag-mediated VLP production by 39% compared to untreated cells (p < 0.01), whereas SptaseIVΔ1 had a minimal influence on VLP production (9% reduction; p > 0.10) (Fig. 2B). Together, these data indicate that PM localization of RSV Gag and efficient VLP assembly require normal intracellular levels of PI(4,5)P$_2$ and PI(3,4,5)P$_3$. However, the effect of SptaseIV expression on RSV budding was less than that observed for HIV-1 Gag, suggesting that additional electrostatic interactions other than PI(4,5)P$_2$ and PI(3,4,5)P$_3$ may contribute to RSV particle assembly.

The NC domain of Gag mediates Gag-Gag and Gag-RNA interactions, and RSV Gag NC domain truncation mutants do not localize to the PM or produce VLPs efficiently (35, 38). However, both PM localization and VLP assembly can be rescued by replacement of NC with a leucine zipper protein-protein dimerization motif from CREB1 (39) (pMA-CA-Zip, also called Gag.Zip; a gift of V. Vogt, Cornell University). In QT6 cells, GagΔNC-YFP (Fig. 3A) (40), which lacks the ability to oligomerize or bind RNA, was diffusely cytoplasmic (M = 0%, C = 100%, n = 22) and was completely unaffected by expression of SptaseIV or SptaseIVΔ1 (Fig. 3B).

In contrast, Gag.Zip-YFP (Fig. 3A) was localized in the PM in a majority of cells (M = 89%, C = 11%, n = 33) and formed long patches along the PM rather than distinct foci (Fig. 3C). Interestingly, the signal from Gag.Zip-YFP overlapped less with PH-mCherry than wild-type Gag (M$_1$ = 0.16 ± 0.03, p = 0.01; M$_2$ = 0.38 ± 0.04, p = 0.02; n = 10), suggesting that either Gag.Zip competes with PH-mCherry for binding to PI(4,5)P$_2$ or Gag.Zip PM binding is independent of PI(4,5)P$_2$. To discriminate between these possibilities, we tested the sensitivity of Gag.Zip to SptaseIV expression. Strikingly, Gag.Zip-YFP was cytosolic with SptaseIV expression in every cell analyzed (M = 0%, C = 100%, n = 34; p < 0.0001) (Fig. 3D), a significant increase compared to the effect of PI(4,5)P$_2$/PI(3,4,5)P$_3$ depletion on wild-type Gag-GFP PM localization (p < 0.002). Expression of SptaseIVΔ1 also reduced the proportion of cells in which Gag.Zip-YFP was PM localized (M = 30%, C = 41%; n = 30; p < 0.05), although the magnitude of the change was similar to that of wild-type Gag-GFP (p > 0.50). Thus, replacing the NC domain with a leucine zipper may make Gag more dependent on PI(4,5)P$_2$ and/or PI(3,4,5)P$_3$ for PM localization despite the presence of a wild-type MA domain.

We next tested whether alterations of the RSV Gag MBD would change the sensitivity of Gag to depletion of intracellular levels of PI(4,5)P$_2$ and/or PI(3,4,5)P$_3$. To this end, we examined the localization of Gag.MyriE, in which the membrane-binding domain of the v-Src oncoprotein was added as an extension at the N terminus of RSV Gag (Fig. 4A) (41). The v-Src MBD is myristoylated and...
FIG 4 Effect of 5ptaseIV expression on RSV Gag.Myr1E-GFP localization. (A) Schematic representation of RSV Gag.Myr1E-GFP showing the 10-amino-acid membrane-binding domain of the v-Src protein as a gray box and myristoylation as a zigzag line. (B) QT6 cells were transfected with pGag.Myr1E-GFP and PH-mCherry. Mander’s colocalization analysis was conducted as described in Fig. 1B. (C) QT6 cells were transfected with pGag.Myr1E-GFP alone (top row) or cotransfected with pmyc-5ptaseIV (middle row) or pmyc-5ptaseIVΔ (bottom row). Graphs on the right show the percentage of cells with Gag in the cytoplasm (C) or at the plasma membrane (M). Statistically significant differences in Gag localization were determined as described in Fig. 2A.

The aim of this study was to determine whether phosphoinositides at the PM are involved in directing RSV Gag to the PM for particle assembly. All of the wild-type and mutant Gag proteins tested were targeted to a similar location as PH-mCherry, which binds to PI(4,5)P$_2$ at the inner leaflet of the PM (Fig. 1B, 3C, 4B, and 5E). However, our finding that depletion of PI(4,5)P$_2$ and PI(3,4,5)P$_3$ results in cytoplasmic Gag accumulation and a moderate reduction in VLP assembly is in contrast to those published by Chan et al. (27). Although the reasons for this discrepancy are not entirely clear, there were some differences in how the experiments were conducted that may provide an explanation. For example, we were careful to limit cellular toxicity of 5ptaseIV expression in our experiments to ensure that the cells remained healthy enough to observe the effect of 5ptaseIV expression on Gag trafficking and VLP production. We transfected 0.5 µg of 5ptaseIV plasmid DNA and we imaged the transfected cells within 15 h of transfection, whereas Chan et al. used 2 µg of DNA for transfection and analyzed cells at 20 to 24 h posttransfection. Moreover, we performed our studies in an investigator-blinded fashion to avoid bias, and experiments were statistically analyzed to ensure reproducibility. Finally, we performed budding assays after a 2.5-hour collection of radiolabeled, immunoprecipitated Gag-GFP proteins with quantitative analysis. In contrast, performing immunoblots of VLPs collected for 24 h (27) may limit the sensitivity

The MA domain was not altered in this case. Instead, the basic residues KKKR in the NC domain, implicated in binding to RSV genomic RNA (44, 45), were replaced by alanine residues (Gag.NC.M1) (Fig. 5A). We previously reported that these basic residues constitute an NLS in NC (21, 46, 47), so we examined whether the loss of the KKKR residues would reduce the nuclear localization of Gag.NC.M1 in the presence of a point mutation in p10 that disrupts the NES. Gag.L219A-YFP (Fig. 3A) accumulated in the nucleus as previously reported (Fig. 5B) (23, 40), whereas Gag.L219A.NC.M1-YFP remained at the PM, indicating that the KKKR residues in NC are required for Gag nuclear import (Fig. 5B). Furthermore, when the NC.M1 mutation was introduced into the proviral vector pRS.V8 (35, 48) and expressed in QT6 cells, genomic RNA packaging was reduced to 10% of the wild-type level with no effect on budding (Fig. 3C and D).

Analysis of Gag.NC.M1-YFP localization indicated partial overlap with PH-mCherry at the PM (M$_1$ = 0.28 ± 0.04; M$_0$ = 0.53 ± 0.03) (Fig. 5E), and the mutant protein was PM localized in nearly all cells (M = 98%, C = 2%, n = 43) (Fig. 5F). Although 5ptaseIV coexpression relocated Gag.NC.M1-YFP away from the PM (M = 64%, C = 36%, n = 49; p < 0.0001) (Fig. 5F), the mutant was less sensitive to 5ptaseIV activity than wild-type Gag-GFP (p < 0.01). Interestingly, Gag.NC.M1 was slightly more sensitive to 5ptaseIV than was Myr1E.Gag-GFP (p < 0.05), possibly due to the increased hydrophobic or electrostatic interaction of the myristoylated Myr1E.Gag N terminus with the membrane. However, Gag.NC.M1-YFP was largely unaffected by 5ptaseIVΔ1 expression (M = 87%, C = 13%, n = 60; p > 0.05) (Fig. 5F). The observation that Myr1E.Gag and Gag.NC.M1 are both deficient in nuclear trafficking and relatively insensitive to 5ptaseIV-mediated depletion of PI(4,5)P$_2$ and PI(3,4,5)P$_3$ suggests an intriguing link between Gag nuclear localization and phosphoinositide-mediated PM localization. In support of this idea, those Gag proteins that do undergo nuclear trafficking (wild type and Gag.Zip) (22, 40) were more sensitive to 5ptaseIV treatment.

Addition of this sequence results in stronger PM binding and enhancement of budding compared to those of the wild-type Gag protein (35, 41). In QT6 cells, Myr1E.Gag-GFP localized near the same sites at the PM as PH-mCherry, and this Gag variant was PM associated in 100% of cells examined (n = 24) (Fig. 4B). However, Myr1E.Gag-GFP localization was largely unaffected by 5ptaseIV (M = 88%, C = 13%, n = 24; p > 0.20) (Fig. 4C) and was significantly less sensitive to 5ptaseIV than wild-type Gag (p < 0.0001). Myr1E.Gag-GFP localization was also unaffected by 5ptaseIVΔ1 expression (M = 100%, C = 0%, n = 25) (Fig. 4C). These results suggest that Myr1E.Gag-GFP either does not interact with PI(4,5)P$_2$ or PI(3,4,5)P$_3$ to the same degree as wild-type Gag or additional mechanisms (e.g., hydrophobic interaction of myristate and/or electrostatic interactions with other negatively charged lipids) stabilize membrane binding, making the mutant protein independent of phosphoinositide levels once it reaches the PM.

Myr1E.Gag exhibits reduced nuclear trafficking, and when expressed in a proviral context, the Myr1E viral mutant (RC.MyrlE) is impaired in genomic RNA incorporation (22, 41). We recently identified another Gag mutant with similar properties, although it consists of 10 amino acids enriched in basic residues (42, 43).
of the assay given that the half-time of budding for RSV Gag is approximately 30 min (23).

Additional evidence that our experimental conditions did not appear to cause nonspecific mislocalization of PM-bound proteins or excessive cellular toxicity was provided by the finding of variable degrees of 5ptaseIV sensitivity among different Gag mutants. Interestingly, RSV Gag.Zip-YFP was more sensitive to 5ptaseIV expression than wild-type Gag, suggesting that that mutant is more dependent on PI(4,5)P$_2$ and/or PI(3,4,5)P$_2$ for stable membrane binding or that a conformational change in the mutant protein modifies the way it binds to the PM compared to the wild-type protein. In either case, the behavior of Gag.Zip-YFP suggests that RNA-driven multimerization of Gag may facilitate attachment to the PM by a different mechanism than those interactions driven by protein–protein oligomerization. Thus, it is possible that RNA interactions influence RSV Gag PM targeting and binding, as suggested for HIV-1 Gag (11).

Interestingly, our data indicate that adding the v-Src membrane-binding domain to Gag results in membrane binding that is less sensitive to depletion of PI(4,5)P$_2$ and/or PI(3,4,5)P$_2$ (Fig. 4C), potentially due to additional hydrophobic or electrostatic interactions of the Gag mutant with the PM. Although v-Src and HIV-1 Gag have similar MBDs composed of N-terminal myristic acid plus nearby basic residues, they differ markedly in their mechanism of membrane interaction, with HIV-1 Gag being much more sensitive to PI(4,5)P$_2$ levels for stable membrane binding than v-Src. These findings are consistent with those reported by Ono et al., who found that a chimeric Fyn HIV-1 Gag construct was markedly less sensitive to 5ptaseIV expression (30), likely due to strong hydrophobic interactions of its myristic acid and palmitic acid moieties with the PM.

Unexpectedly, we found that altering the NLS in NC caused the mutant Gag.NC.M1 protein to be strongly localized to the PM and reduced in its sensitivity to 5ptaseIV treatment. This finding was surprising because we had not previously identified a mutant in the NC domain that altered trafficking to the PM. Furthermore,
we expected the remaining NLS in MA to retain nuclear trafficking activity even in the absence of the KKKR NLS in NC, especially given that a mutant having a deletion of the entire NC sequence still traffics through the nucleus (21). Thus, it is possible that mutating the basic residues in NC alters the global conformation of Gag, inducing a structural rearrangement of the MBD that eliminates MA NLS activity while simultaneously strengthening PM binding. It is plausible that Gag.NC.M1 may adopt an “extended” conformation (49) that favors PM targeting and interferes with binding of nuclear import factors to the NLS in MA.

Gag.NC.M1 and Myr1E.Gag are both impaired in nuclear trafficking, and their membrane-binding mechanisms are less dependent on Sp5aseIV expression, suggesting a potential link between nuclear localization of Gag and PI(4,5)P2/PI(3,4,5)P3-mediated PM binding. We find this possibility to be quite intriguing because PI(4,5)P2 is present in the nucleus and has been implicated in mRNA processing, mRNA export, chromatin remodeling, and transcriptional regulation (50–57), whereas nuclear PI(3,4,5)P3 has been linked to the inhibition of apoptosis through its interaction with the nuclear protein B23 (58). Thus, we propose the hypothesis that Gag may recruit nuclear PI(4,5)P2 or PI(3,4,5)P3 into the Gag-genomic RNA complex in the nucleus or nucleolus, as we have recently demonstrated nuclear trafficking of RSV Gag (47). After exiting the nucleus through the nuclear pore, PI(4,5)P2 or PI(3,4,5)P3 may play a role in directing the viral ribonucleoprotein complex to the PM for final assembly of the virus particle. Because they bypass the nucleus, Gag.NC.M1 and Myr1E may not interact with nuclear phosphoinositides, altering the mechanisms by which these mutant proteins traffic to and bind to the PM. We also consider the possibility that other nuclear factors may be involved, and future studies will aim to identify host factors involved in the intracellular transport of the RSV Gag ribonucleoprotein complex from the nucleus to the PM for virion assembly.

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Phosphoinositide-Dependent Localization of RSV Gag


Appendix C

Nucleolar Localization of HIV-1 Gag

Figure C.1 adapted from:
171(2): 304 – 318

**Abstract**

In Chapter 2 we reported the nuclear and nucleolar localization of a population of MMTV Gag under steady-state conditions, and that nucleolar localization of Gag was increased with overexpression of RPL9. At the same time, other experiments in our lab demonstrated that RSV Gag could also accumulate in nucleoli when Gag was trapped in the nucleus. Furthermore, a population of HIV-1 Gag also accumulated in the nucleolus under certain conditions. As a result, we sought to determine whether HIV-1 Gag was also present in the nucleolus under steady-state conditions. Using stable cell lines containing doxycycline-inducible HIV-1 proviruses we found that 2.76±0.12% of HIV-1 Gag localized to the nucleolus under steady-state conditions, indicating that Gag proteins from many retroviruses may transiently traffic through the nucleolus.
Gag proteins of several retroviruses traffic through, or partially localize to the nucleus [94,251,414,415,416,417,418], however sub-nuclear localization of Gag is less well-documented. We found that a population of MMTV Gag localizes to the nucleolus under steady-state conditions and that overexpression of the large ribosomal subunit protein RPL9 increased nucleolar localization of Gag (Chapter 2). At the same time that these experiments were being conducted, other experiments in our lab revealed that RSV Gag could localize to nucleoli when Gag was trapped in the nucleus by treatment with the Crm1 inhibitor Leptomycin B, or when the nuclear export signal was mutated. Strikingly, HIV-1 Gag also accumulated in the nucleolus when co-expressed with the NC domain of HIV-1 Gag or the HIV-1 Rev protein [421]. Together, these data suggested that nucleolar trafficking may be common to many retroviral Gag proteins. However, it was not known whether HIV-1 Gag was also present in the nucleolus under steady-state conditions.

To examine the nucleolar localization of HIV-1 Gag, we utilized two HeLa cell lines containing integrated HIV-1 proviral constructs: HIV Δmls rtTA [458] and HIV.Gag-GFP rtTA. Both constructs lack protease and the reverse transcriptase portion of the Pol gene, and provirus expression is dependent on the addition of doxycycline to the culture media (Fig. C.1A). Furthermore, these proviruses recapitulate authentic HIV-1 because Gag expression is completely dependent upon Rem [458]. To visualize nucleolar localization of Gag, provirus expression was induced by adding 2 µg/mL of doxycycline to the culture media for 24 hours. Cells were then fixed in 4% paraformaldehyde for 30 minutes, permeabilized with
Figure C1. A subpopulation of HIV-1 Gag localizes to nucleoli. (A) A cartoon showing the HIV Gag-GFP rtTA construct used to create a HeLa cell line stably expressing HIV Gag-GFP under the control of a doxycycline promoter. (B) Cells expressing HIV Gag-GFP rtTA or (C) HIV Δmls rtTA were prepared for imaging 24 hours after adding doxycycline to the culture media. Cells were imaged through the largest diameter of the nucleus using a Leica SP2 confocal microscope. In (B) Gag localization was determined by direct visualization of GFP and nucleoli were identified by immunostaining for B23, while cells in (C) were immunostained for Gag and EBP2 to identify nucleoli. The intensity of the images in the middle panels was increased to better show Gag fluorescence in nucleoli.
A

HIV.Gag-GFP rTA

B

HIV.Gag-GFP rTA

Gag-GFP  ↑ Intensity  anti-B23 (Texas Red)

C

HIVΔms rTA

anti-p24 (FITC)  ↑ Intensity  anti-EBP2 (Texas Red)
1% Triton X-100 for 5 minutes and blocked with 3% goat serum. In HIV.Gag-GFP rtTA cells, nucleoli were identified by immunostaining with rabbit anti-B23 (a gift from L. Frappier, University of Toronto) and anti-rabbit-Texas Red secondary antibody (Jackson Immunoresearch). By contrast, HIV Δmls rtTA cells were immunostained with mouse anti-HIV CA (183, a kind gift from M. Tremblay, Laval University) and anti-mouse FITC antibody, then counterstained with rabbit anti-hEBP2 (a kind gift from L. Frappier, University of Toronto) and anti-rabbit Texas Red (Jackson Immunoresearch). Cells were imaged using a Leica SP2 ABOS Confocal microscope through the largest diameter of the nucleus. Nucleoli were identified by the localization of B23 or hEBP2, and the amount of Gag or Gag-GFP signal present in the nucleolus compared to the entire cell was calculated using the “measure” tool in ImageJ [361].

In the absence of doxycycline, no HIV-1 Gag expression was detected from HIV.Gag-GFP rtTA cells (data not shown) [458]. However, addition of doxycycline induced Gag expression and a subpopulation of Gag colocalized in nucleoli with B23 (Fig. C.1B). Analysis revealed that approximately 2.76±0.12% of the total Gag signal within the cell was localized to nucleoli, indicating that a small but significant population of Gag is present in nucleoli under steady-state conditions. Treatment with leptomycin B had no effect on Gag nucleolar localization, consistent with previous reports [251]. This nucleolar localization of Gag-GFP is unlikely to be derived from free NC-GFP because the HIV.Gag-GFP rtTA clone lacks viral protease, and SDS-PAGE and Western blotting experiments revealed that full-length Gag-GFP is, by far, the most predominant protein species present
in HIV.Gag-GFP rtTA cells stimulated with doxycycline (data not shown). Furthermore, similar results were obtained in HIV Δmls rtTA cells immunostained for Gag using an anti-CA antibody, suggesting that full-length Gag is present within nucleoli (Fig. C.1C). As a result, these data indicate that a small population of Gag is present in nucleoli when expressed in a Rev-dependent context.

Although the biological relevance of this finding remains to be determined, several lines of evidence suggest that nucleoli may play an important role in HIV-1 replication. Primarily, data indicate that HIV-1 genomic RNA may be present in nucleoli, and furthermore, HIV-1 Rev interacts with B23 to localize to nucleoli where it dimerizes and binds HIV-1 RNA [241,459,460]. The observation that Rev expression redistributes Gag to nucleoli [421], combined with reports that Rev may play a role in gRNA encapsidation [151,392], suggests that Rev may help localize HIV-1 Gag to nucleoli to bind and encapsidate gRNA. Furthermore, the observation of Gag proteins from four different retroviruses (HIV, RSV, FIV, and MMTV) in nucleoli indicates that nucleolar trafficking of Gag may be relatively conserved among retroviruses [251,421] (Chapter 2).
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Vita

Darrin V. Bann

Academic Degrees
Ph.D. in Cellular & Molecular Biology 2009 – 2013
   The Pennsylvania State University, College of Medicine (Hershey, PA)
M.D. 2007 – 2015
   The Pennsylvania State University, College of Medicine (Hershey, PA)
   Ithaca College (Ithaca, NY)

Selected Abstracts

Selected Publications
*authors contributed equally
*authors contributed equally
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