Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 1/1/2010 - 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John Anthony, MPA

4. **Grant Contact Person's Telephone Number:** 814 935 1081

5. **Grant SAP Number:** 4100050904

6. **Project Number and Title of Research Project:** 34. Viral RNA Trafficking in Living Cells

7. **Start and End Date of Research Project:** 9/1/2010 - 12/31/2013

8. **Name of Principal Investigator for the Research Project:** Leslie J. Parent, MD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 192,390

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Bann	Graduate Assistant	74% Yr 1; 6% Yr 2	$23,055.99
Rice	Graduate Assistant	25% Yr 3; 5.2% Yr 4	8,989.93
Bjanes	Undergrad Summer Intern	100% Yr 3	3,238.60

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Parent, Leslie	Principal Investigator	2%
Bann	Grad Student – MD/PhD Program	14%, Yr 2; 100%, Yr 3 100%, Yr 4
Lambert	Summer Undergraduate Intern	100%, Yr 3; 100%, Yr 4

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Imaris software	Used for analysis of microscopic images (subcellular localization, quantitation of colocalization, 3D reconstructions, etc.)	$24,844
Tokai Hit Stage Top Incubator	Used for live cell imaging and time courses in conjunction with Leica SP8 confocal microscope.	$6,654
BioRad gel imaging system	Used for quantitative analysis of Western Blots and for imaging DNA in agarose gels	$41,912

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes____X_____ No_____

If yes, please indicate the source and amount of other funds:

Darrin Bann received an NIH F30 award, which supported his stipend and tuition for the part of year 2 and all of years 3 and 4. The total amount of funds supported by the NIH F30 award during the project period was $41,907.97. Supplemental stipend funds of $2,242.48 were

paid from Medicine general funds during the project period. Also during the project period, the College of Medicine MD/PhD program general funds paid Darrin's fall 2013 MD tuition of $25,992 not covered by the NIH F30 award.

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
R21 Interplay Between Retroviruses and Retrotransposons	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	February 2012	$275,000 direct costs; $416,850 total costs	$ 0

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

I plan to resubmit the R21 application described above, which received a priority/impact score of 37 on the first submission.

12. Future of Research Project. What are the future plans for this research project?

We are continuing to work on this research project. A manuscript describing the role of Mov10 in MMTV assembly is in preparation. We plan to resubmit the R21 application, as described above.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male			1	
Female	2		1	
Unknown				
Total	**2**		**2**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	2		2	
Unknown				
Total	**2**		**2**	

	Undergraduate	Masters	Pre-doc	Post-doc
White	2		2	
Black				
Asian				
Other				
Unknown				
Total	**2**		**2**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No_____X____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This grant provided funds to purchase important equipment that enhanced this research project and the research of other Penn State researchers.

- Imaris software (Bitplane Inc.): Used for analysis of confocal and deconvolution microscopic images. This software was used in our project to analyze confocal images for colocalization, quantitative subcellular localization, three-dimensional reconstruction, analysis of live cell images, particle tracking, and to make movies. The program is loaded on a computer in the PSU COM Imaging Core Facility so all investigators can use it. (see Bann et al., 2014)
- Tokai Top Hit Stage Incubator: Used to provide a heated stage, incubated stage for confocal live cell imaging on the Leica SP8 microscope. We used this stage to acquire images of living cell expressing MMTV subviral RNA and Gag proteins tagged with fluorescent molecules to study retrovirus assembly. In addition to this project, the equipment is also used by other investigators at the PSU COM for their microscopic studies. (see Bann and Parent, 2012)
- Biorad gel imaging system: Used for quantitative analysis of Western Blots and for imaging DNA in agarose gels. We used the gel imaging system to quantitate our Western blots for siRNA-mediated knockdown and for quantitation of MMTV assembly. The gel imaging system is housed in the Department of Microbiology & Immunology and is used widely by these investigators. (see Bann et al., 2014)

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

Two undergraduate students (Kristen Lambert and Elisabet Bjanes) from the Schreyer Honors College at Penn State University worked on this project during the summer of 2013 as part of the College of Medicine MD/PhD Program and the Summer Undergraduate Research Internship Program.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No___X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Overall description of the project:
This project focuses on the mechanism used by the oncogenic retrovirus MMTV to produce new virus particles in infected mammary cells. We have compelling new evidence that MMTV capsids associate with RNA processing machinery, which is the foundation for our hypothesis: MMTV genomic RNA (gRNA) is targeted to PB/SG/RISCs where it binds to the structural protein Gag to encapsidate the viral genome into assembling capsids. The movement of MMTV Gag and gRNA are dynamic, transient events that can only be revealed using realtime imaging of individual living cells. Therefore, to test our hypothesis, we will develop an innovative method to track MMTV gRNA in live cells using florescence microscopy.

Project goals, objectives and specific aims:
Specific Aim 1: Identify RNA processing factors that mediate MMTV gRNA trafficking in living cells. Following integration of proviral DNA into the host genome, retroviruses produce unspliced viral RNAs that are used for encapsidation into virions (gRNA) or as a template for protein synthesis (mRNA). To initiate capsid assembly, Gag binds the gRNA, then multimerizes to form an immature capsid. Efficient assembly requires that gRNA be separated from viral mRNA and transported to intracellular sites of Gag accumulation. However, the mechanism used to sort gRNA from viral mRNA is unknown. Our preliminary studies suggest that MMTV hijacks the mRNA-silencing pathway to sequester its gRNA into a protected environment, where Gag proteins are localized. Because micro-RNAs (mi-RNAs) bind complementary sequences in mRNAs to suppress their translation, it is feasible that viral or host encoded mi-RNA could serve as a tag to "mark" MMTV gRNA, directing it to PB/RISCs. To test the idea that PBs/SGs/RISCs serve as the staging site for MMTV capsid assembly, we propose this set of experiments:

1a. Track MMTV RNA in living cells to visualize sites of gRNA localization.
1b. Determine the role of PB/SG/RISCs in MMTV gRNA trafficking.
1c. Determine whether the mi-RNA pathway influences MMTV gRNA localization.

We received additional funds that enabled us to establish a new Aim that is consistent with the original goals of the award.

Specific Aim 2: Our preliminary data suggest that MMTV RNA and its structural protein, Gag, localize with cellular mRNA processing factors that form SGs, PBs, and RISCs, where cellular mRNAs are sent for translational repression, storage, and/or degradation. Others have shown that LINE-1 RNA and its structural protein ORF1p also accumulate in RNPs that co-localize with SGs/PBs/RISC proteins. Thus, the cell may try to eliminate retroelement RNAs and repress their translation by targeting the RNA for degradation. However, retroelements may use this sequestration of their RNA in cytoplasmic granules to their advantage by sending their structural proteins (Gag for MMTV and ORF1p for LINE-1) to these sites to bind their RNA genomes in these RNP complexes. Therefore, we hypothesize that mRNA processing sites (SGs/PBs/RISCs) are a battleground between intrinsic host defense designed to restrict expression of foreign RNAs and retroelements that utilize these sites to promote their own replication. The overall goal of the second specific aim is to develop methods to study endogenously expressed LINE-1 and MMTV with the following specific aims:

2a. Develop methods for detecting endogenous LINE-1 activity
2b. Develop a packaging assay to quantitate MMTV genomic RNA in virions
2c. Determine whether components of RNA silencing complexes (RISCs) inhibit LINE-1

retrotransposition and/or MMTV replication.

During the funding period, we have made significant progress in understanding how the MMTV Gag protein and the viral RNA interaction with the SG protein YB-1 to traffic to sites of viral capsid assembly. Studies are underway to determine whether MMTV Gag and murine LINE-1 Orf1 protein interact in the same cytoplasmic granules in MMTV-infected cells.

Progress Report for Aim 1a: Track MMTV RNA in living cells to visualize sites of gRNA localization.

The following progress report was modified from our publication: Darrin V. Bann and Leslie J. Parent, "Tracking Retroviral RNAs in Living Cells," *Viruses.* 2012 Jun;4(6):963-79. doi: 10.3390/v4060963. Epub 2012 Jun 8. Review. PMID: 22816035.

Abstract: Retroviruses produce full-length RNA that serves both as a genomic RNA (gRNA), which is encapsidated into virus particles, and as an mRNA, which directs the synthesis of viral structural proteins. However, we are only beginning to understand the cellular and viral factors that influence trafficking of retroviral RNA and the selection of the RNA for encapsidation or translation. Live cell imaging studies of retroviral RNA trafficking have provided important insights into many aspects of the retrovirus life cycle, including transcription dynamics, nuclear export of viral RNA, translational regulation, membrane targeting, and condensation of the gRNA during virion assembly. Here, we review cutting-edge techniques to visualize single RNA molecules in live cells and discuss the application of these systems to studying retroviral RNA trafficking.

1. Introduction: Recombinant green fluorescent protein (GFP) was first cloned over 25 years ago, permitting fluorescent labeling of proteins for *in vivo* trafficking and localization studies (19, 40). Until recently, there has been no analogous system to visualize RNA trafficking in living cells, so many RNA localization studies have used fluorescently-labeled probes to visualize RNA transcripts in fixed cells. However, these studies only provide a snapshot of RNA localization at a single point in time. On the other hand, live cell RNA tracking studies allow the dissection of each step in RNA metabolism, including transcription, post-transcriptional processing, nuclear export, post-transcriptional regulation, and RNA decay. Accordingly, over the last decade there has been a rapid expansion of methods to visualize single RNA molecules in living cells.

The first experiments to visualize the localization of mRNA molecules in live cells involved microinjection of fluorescently labeled full-length mRNAs (3). However, this approach is time consuming, technically challenging, and may not reflect the trafficking pathways of native transcripts. Techniques used to visualize endogenous RNA rely on the binding of fluorescent oligonucleotides, small molecules, or protein reporters to a specific sequence within the transcript of interest. Using fluorescent molecules to visualize RNA in living cells is limited by the need to deliver the reporter without causing cellular toxicity. This problem can largely be overcome by using sequence-specific RNA binding proteins labeled with a fluorophore such as GFP and titrating expression levels. Furthermore, most RNA tracking systems have been modified or adapted to increase signal-to-noise ratio, allowing the detection of single RNA

molecules over background fluorescence from unbound reporter. As a result, these systems have provided valuable insight into the localization and regulation of cellular RNAs.

In addition to tracking cellular mRNAs, fluorescent RNA labeling techniques are also well suited to study viral RNA trafficking. RNA synthesis is essential for the replication of all viruses, however cellular defensive strategies have evolved to recognize and destroy viral RNA. As a result, viruses have developed diverse mechanisms to facilitate viral replication while circumventing host antiviral defenses. Many, if not all, RNA viruses interact with cellular RNA processing machinery to disable cellular defenses against viral infection or to facilitate viral replication. Therefore, studying the trafficking of viral RNA in living cells may provide insight into how cells defend against viral invasion and how viruses are able to avoid cellular defenses and facilitate their own replication.

Retroviruses are positive stranded RNA viruses characterized by the ability to reverse-transcribe their RNA genomes into DNA and stably integrate into the chromosome. Following integration all retroviruses express full-length RNA that serves two roles in the viral replication cycle: (i) to serve as a viral mRNA to direct the synthesis of the retroviral structural protein, Gag, and (ii) to serve as a genomic RNA (gRNA), which is encapsidated into nascent virus particles. Genome encapsidation is initiated when the gRNA is bound by Gag, however for most retroviruses this process occurs in *trans*, meaning that Gag does not bind the RNA from which it was translated (17). This feature of retroviral replication suggests that there may be a mechanism to spatially separate viral RNAs destined for encapsidation from those to be used for translation. Accordingly, tracking the intracellular fate of full-length retroviral RNAs can provide insights into retroviral RNA trafficking and can serve as a model system to study post-transcriptional regulation of cellular mRNAs. Here, we review some of the most common methods used to visualize single RNA molecules in living cells with a focus on how these techniques can be applied to the study of retroviral RNA trafficking.

2. Hybridization-based RNA Labeling Techniques

Radiolabeled RNA probes were first used to study ribosomal gene amplification in *Xenopus* in 1969 (34). Although the technology available to image specific nucleic acid sequences has improved dramatically over the last 50 years, the same principles apply to hybridization-based approaches to visualizing RNA in cells. In each of these approaches, fluorescently labeled oligonucleotide probes complimentary to the transcript of interest are introduced into live cells. Single molecule sensitivity can be achieved by using several probes targeting the same transcript, or by reducing background signal associated with unbound probe.

2.1 Linear oligonucleotide probes

RNAs can be visualized in living cells using fluorescently-labeled, linear probes complementary to the sequence of interest (Table 1) (79). However, in live cells, unbound probe cannot be removed by washing. As a result, this approach requires the introduction of multiple unique probes targeting the same transcript so that localized concentrations of probes can be differentiated from high levels of background fluorescence (66). Linear probes have been useful in visualizing RNAs associated with small nuclear ribonucleoprotein complexes (snRNPs) and ribosomal RNAs associated with nucleoli (18, 66). However, limitations including low signal-to-noise ratios, the need to microinject probes into cells of interest, and the tendency of probes to

rapidly accumulate in the nucleus following microinjection have discouraged more widespread use of this approach (8).

To increase the signal-to-noise ratio associated with linear probes, some groups have utilized Förster resonance energy transfer (FRET)-based systems (98) (75). In this approach, two probes complementary to adjacent regions on the transcript of interest are labeled with donor and acceptor fluorophores, respectively. Hybridization of each probe to the transcript brings the fluorophores into close proximity, resulting in FRET when the donor fluorophore is excited **(**Table 1**)** (98). Both probes must bind the transcript of interest to produce a FRET signal, increasing the specificity of this system over single linear probes (98). The use of FRET also increases the signal-to-noise ratio of the system, because FRET only occurs when both probes are bound to the same transcript. The FRET signal can be further increased through the addition of multiple donor fluorophores (76). Alternatively, background FRET signal can be reduced through the use of autoligation FRET probes. In this system, the donor probe is labeled with a fluorophore and a quencher, preventing fluorescence of the donor. Hybridization of the donor and acceptor probes to the transcript results in autoligation of the donor and acceptor probes with excision of the quencher, producing a FRET signal (Table 1) (1, 103). While FRET-based systems may overcome some of the sensitivity issues associated with single linear probes, they share the same delivery obstacles. As a result, these systems have not been used extensively to visualize viral RNA in living cells.

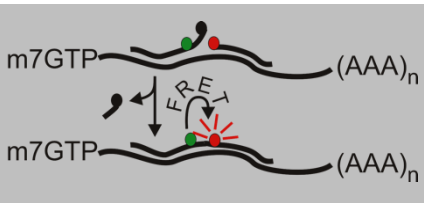
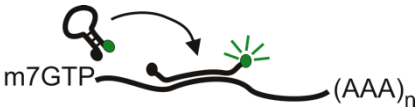
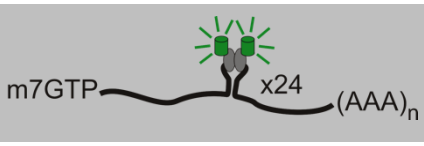
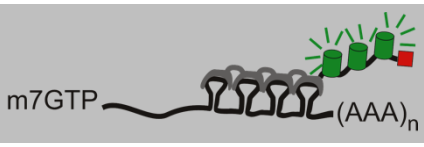
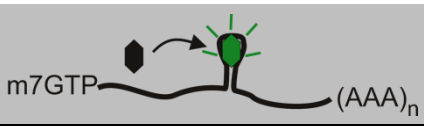
2.2 Molecular Beacons

Unlike linear probes, molecular beacons form stem-loop structures. The 5' and 3' ends of the beacon form a self-complimentary stem, while the central loop region is complimentary to the transcript of interest (99). The 5' and 3' ends of the molecule are conjugated to a fluorophore and a quencher, which prevents fluorescence when the beacon is in a stem-loop conformation. Binding of the beacon to the transcript induces melting of the molecular beacon stem structure, moving the fluorophore away from the quencher and resulting in fluorescence (Table 1). A further increase in the signal-to-noise ratio associated with molecular beacons can be achieved using FRET between two molecular beacons labeled with donor and acceptor fluorophores (92). Owing to the decreased background fluorescence associated with molecular beacons compared to linear probes, molecular beacons have been used more extensively to visualize viral RNAs in living cells. This approach has been used to monitor the spread of bovine and human respiratory syncitial viruses (90, 91)and coxsackievirus B6 (106) in tissue culture, as well as to study the intracellular trafficking dynamics of poliovirus positive-stranded RNA (25), and influenza A virus mRNA (100).

To study viral RNA trafficking, molecular beacons offer some advantages over the fluorescent protein-based RNA labeling techniques discussed below. Primarily, because molecular beacons hybridize to a sequence of interest, this approach can be used to visualize native (i.e. non-engineered) viral RNAs. As a result, molecular beacons can be used to visualize viral RNAs in clinical isolates (90), obviating the need to introduce a foreign RNA sequence into the viral RNA of interest. However, the use of molecular beacons to study retroviral RNA trafficking may be complicated by the presence of endogenous proviruses, which frequently share a high degree of sequence homology with exogenous retroviruses. For example, many strains of laboratory mice express up to eight endogenous retroviruses with high homology to exogenous mouse mammary

tumor virus (MMTV) (50). Consequently, trafficking experiments could be confounded by the inability of molecular beacons to effectively distinguish exogenous retroviral RNA from RNA expressed by endogenous retroviruses.

Table 1. Methods to visualize RNA in living cells.

Method	Diagram	Recognition Sequence	Citations
Linear Oligonucleotide Probe		User defined	(18, 66)
Linear FRET Probe		User defined	(75, 98)
Autoligation FRET Probe		User defined	(1, 103)
Molecular Beacon		User defined	(99)
MS2-XFP		19 nucleotide stem-loop	(12, 33)
Bgl-XFP		29 nucleotide stem-loop	(22)
λ_N-GFP		15 nucleotide stem-loop	(26)
PUM-HD		User defined	(77, 104)
Spinach		Varies based on desired fluorescence	(78)

An additional obstacle to the use of hybridization-based probes, including molecular beacons, is the delivery of probes into living cells. Microinjection is the most direct delivery method and allows for very rapid visualization of target RNAs (62), but poses technical challenges.

Accordingly, other methods are frequently used to deliver molecular beacons into the cells. One approach is to reversibly permeabilize cells using streptolysin O (90, 100). This technique offers rapid visualization of target RNAs without the technical requirements of microinjection (79).

Rapid introduction oligonucleotide probes or molecular beacons into cells can also be accomplished by conjugating the probe to a cell-penetrating peptide, including HSV-1 VP22 or HIV-1 Tat (8). Alternatively, cationic lipid-based transfection reagents have been used to deliver molecular beacons into cells (25). Unlike permeabilization or cell-penetrating peptides, however, these reagents may deliver molecular beacons to the endocytic pathway, resulting in nuclease degradation of the beacon and increased background fluorescence (8). However, non-specific fluorescence associated with molecular beacon degradation can be reduced using 2'O-methylated probes (66) or by using specific quantum dot-molecular beacon conjugates (21). Other transfection methods such as electroporation have also been used to introduce molecular beacons into live cells, though all transfection-based delivery methods impart a lengthy delay before target transcripts can be visualized (8). As a result, molecular beacons and other hybridization-based techniques can be powerful tools to track viral RNAs in living cells, but all of these approaches share some common limitations and are not universally applicable to every model system.

3. RNA Binding Protein-Based RNA Labeling Techniques

The use of oligonucleotide probes presents many challenges to live-cell imaging of single RNA molecules. To circumvent some of these problems, plasmid-based systems have been developed using fluorophore-labeled RNA binding proteins that specifically recognize RNA sequences inserted into the transcript of interest. Alternatively, fluorophore-labeled cellular RNA-binding proteins can be mutated to recognize specific RNA sequences, permitting the visualization of endogenous transcripts.

3.1 MS2-GFP

The MS2 RNA-binding protein forms the viral capsid of the MS2 bacteriophage. During the late phase of phage replication, an MS2 dimer binds a 19-nucletide stem-loop structure on the phage genomic RNA, which initiates phage capsid assembly and encapsidation of the genome (73). In 1998, Bertrand *et al*. took advantage of this system to visualize RNA localization by using an MS2 mutant that binds RNA but is incompetent for capsid assembly (83). A nuclear localization signal (NLS) was added to the MS2-GFP fusion protein to reduce background fluorescence in the cytoplasm. To visualize RNA localization in yeast, six MS2 stem-loops were inserted between the *LacZ* coding region and the *ASH1* 3' untranslated region (*LacZ-MS2-ASH1*) (Table 1) (12). When MS2-GFP was expressed alone, the fluorescent signal accumulated in the nucleus by virtue of the NLS. However, when MS2-GFP was coexpressed with *LacZ-MS2-ASH1*, discrete MS2-GFP foci were visualized in the nucleus and cytoplasm. Subsequent refinements demonstrated that inserting 24 copies of the MS2 stem-loop sequence into the RNA yielded single-molecule sensitivity (33). Interestingly, on average only 33 molecules of GFP were associated with each RNA (33). Because MS2 binds as a dimer, this result suggests that on

average only about two-thirds of the 24 MS2 stem-loops were occupied by MS2-GFP. In fact, Fusco *et al*. (33) visualized single RNA particles containing as few as 20 GFP molecules, indicating that single RNAs can be visualized using smaller numbers of fluorophores.

The use of MS2-GFP offers several advantages to hybridization-based approaches; most notably RNA can be tracked in living cells, obviating the concern for fixation artifacts. The high affinity interaction between the MS2 coat protein and the MS2 RNA (Kd = 0.4 nM) results in specific labeling of RNAs containing the MS2 stem loops (53). The presence of the NLS on MS2-GFP allows newly synthesized RNA to be bound by the reporter co-transcriptionally (14, 93), resulting in a high signal-to-noise ratio in the cytoplasm (33). This system does, however have the disadvantage that MS2-GFP only binds to RNAs engineered to contain MS2 stem-loops, so it cannot be used to track native RNAs. Furthermore, achieving an optimal ratio of MS2-GFP to RNA expression may require titrating different amounts of each expression construct (12). The localization of free MS2-GFP in the nucleus may partially obscure visualization of nuclear RNAs, although the punctate intranuclear MS2-GFP signal can be brought out by carefully titrating MS2-GFP expression [37(14, 58) and our unpublished data]. Another potential concern arises from the possibility that the RNA-bound MS2-GFP protein could alter normal RNA trafficking. However, RNAs bound by MS2-GFP are efficiently exported from the nucleus and properly localized in the cytoplasm (33, 68, 69). Furthermore, retroviral RNAs bound by MS2-GFP are efficiently encapsidated into virus particles [(22) and our unpublished data]. Together, these data suggest that MS2-GFP does not interfere with proper RNA trafficking or binding by the retroviral Gag protein.

Due to its ease of use and relatively few drawbacks, the MS2-GFP system has been used to study retrovirus RNA biology in living cells. Boireau and colleagues (14) took advantage of the high affinity interaction between MS2-GFP and HIV-1 RNAs containing 24 tandem copies of MS2 stem-loops to study the kinetics of HIV-1 RNA transcription using fluorescence recovery after photobleaching (FRAP). Nuclear MS2-GFP was subjected to photobleaching, and fluorescence recovery occurred when free MS2-GFP proteins bound to newly transcribed RNAs bearing the MS2 stem-loops (14), allowing measurement of the rate of HIV-1 RNA transcription. In addition, due to very slow exchange of RNA-bound MS2-GFP with free MS2-GFP, this system can be used to track single RNA molecules over time. By using a photoactivatable form of GFP, RNA molecules can be differentially labeled at sites of transcription (38, 93). In theory, this approach could allow the tracking of single RNA molecules throughout the entire process of retrovirus particle formation.

The MS2-GFP system has also provided valuable insight into the viral and cellular factors that regulate the trafficking of retroviral RNAs. The canonical view of retroviral assembly holds that for C-type retroviruses, Gag and the gRNA interact at the plasma membrane to assemble new virus particles. However, using MS2-GFP, Gag-RNA interactions have been visualized at earlier locations in the assembly pathway. For example, the feline immunodeficiency virus (FIV) Gag protein co-localizes with the gRNA on the cytoplasmic face of the nuclear envelope (49), whereas HIV-1 and murine leukemia virus (MLV) Gag-gRNA interactions were visualized on the cytoplasmic face of endosomes (9, 49). The high sensitivity of the MS2-GFP system has also permitted a detailed kinetic analysis of individual HIV-1 virus particle formation at the plasma

membrane (41). Together, these data have helped solidify the conclusion that retroviral gRNA is bound by Gag prior to localization of the viral nucleoprotein complex at the plasma membrane.

Another informative application of the MS2-GFP system is to visualize protein-RNA and RNA-mediated protein-protein interactions in living cells. This approach, called trimolecular fluorescence complementation (TriFC), utilizes MS2 conjugated to a non-fluorescent N-terminal fragment of Venus fluorescent protein (MS2-VN). Binding of an MS2 stem-loop containing RNA by MS2-VN and a target protein containing the C-terminal fragment of Venus (VC) results in reconstitution of the Venus fluorophore and detectable fluorescent signal (84). Recently, Milev *et al*. (63) used TriFC to probe Gag-Gag and Gag-Staufen interactions on viral RNA. As expected, Gag-VC complemented MS2-VN on *psi*-containing RNAs, however Staufen-VC failed to complement MS2-VC in the absence of Gag (63). To examine whether Gag could recruit Staufen to viral RNAs, the authors took advantage of the fact that the MS2 protein binds to RNA as a dimer. By co-expressing MS2-Gag constructs with MS2-VN and VC-labeled target proteins, they found that HIV-1 Gag actively recruits Staufen to the viral RNA (63). This study demonstrates that MS2-GFP is a powerful system to study the dynamic trafficking of viral RNAs and to probe host-virus interactions in living cells.

3.2 Bgl-mCherry

The Bgl-mCherry RNA labeling system, analogous to the MS2-GFP system, is derived from the *E. coli* anti-termination protein BglG. The first 58 amino acids comprise the BglG RNA-binding domain, and this protein fragment is sufficient for anti-termination activity (59). To create a live-cell RNA labeling system, Chen *et al*. (22) fused the N-terminal 52 amino acids of the BglG RNA-binding domain to mCherry and an NLS to create Bgl-mCherry. Bgl-mCherry dimers bind a 29-nucleotide stem-loop structure, and single molecule sensitivity has been obtained using only 18 Bgl stem-loops (Table 1) (22). However, it is not clear whether all 18 stem-loops are required to achieve this level of sensitivity.

This system shares many of the advantages and disadvantages of the MS2 RNA labeling system. Bgl-mCherry binds with high affinity to RNAs containing BglG stem-loops, allowing selective labeling of the target RNA (22). Also like MS2, the multiple, highly repetitive stem loop sequences can be unstable, although this problem can be managed by using recombination-deficient bacterial strains. The Bgl-mCherry system has been used successfully to label HIV-1 RNAs, and viral genomes bound by Bgl-mCherry are incorporated into retrovirus particles with >90% efficiency, suggesting that Bgl-mCherry is unlikely to adversely affect viral RNA trafficking (22).

Due to the highly specific binding of MS2-GFP and Bgl-mCherry to their respective RNA sequences, separately labeled RNAs can be discriminated within the same cell (22, 28). Moreover, because MS2-GFP and Bgl-mCherry are efficiently encapsidated into retrovirus particles, the genomic RNA content of single virions can be compared (22, 28, 68, 74). The Hu laboratory has used this system to study genome encapsidation by HIV-1. Because retroviral genomic RNAs (gRNAs) are encapsidated as a non-covalent dimer, packaging of two heterologous RNAs can lead to recombination during reverse transcription (38). When *psi*-containing HIV-1 RNAs labeled with MS2-GFP or Bgl-mCherry were co-expressed, the genomic RNAs were randomly assorted into virus particles with the expected 1:2:1 ratio (22).

However, mutations within the genomic RNA dimerization initiation sequence disrupt this ratio, suggesting that dimerization occurs prior to encapsidation (22). Using the same system, they also demonstrated that genome dimerization appeared to occur in the cytoplasm and that the RNA nuclear export pathway influences the assortment of genomic RNAs virus particles (68). The Bgl-mCherry system has also been used to study encapsidation of HIV-2 RNA by HIV-1 Gag (28) and to study the mechanism of encapsidation of genomic RNA by HIV-2 (74). Other retroviruses, including MMTV and Mason-Pfizer Monkey Virus (MPMV), can cross-package the genomes of orthologous retroviruses (4). Furthermore, exogenous retroviruses can recombine with endogenous retroviruses to create novel pathogens (42). As a result, single particle RNA analysis using MS2-GFP and Bgl-mCherry may provide valuable insight into the frequency and mechanisms of cross-packaging events and may shed light onto the mechanisms used by different retroviruses to select genomic RNAs for packaging.

3.3 λ_N-GFP

One of the difficulties associated with the MS2-GFP and Bgl-mCherry systems is the insertion of long (e.g., ~1,300 nucleotides for 24 MS2 stem-loops), highly repetitive sequences into the RNA of interest. To circumvent this problem, Daigle and Ellenberg recently developed the λ_N-GFP system to study the intracellular trafficking of MMTV RNA (26). The λ_N-GFP reporter protein is composed of four tandem repeats of the N-terminal 22 amino acids of the bacteriophage λ N protein (λ_N) fused to three eGFP molecules and an M9 localization signal. The λ_N-GFP reporter binds to four 15-nucleotide stem-loop structures called BoxB, which are inserted into the target RNA (Table 1) (26). Each set of four BoxB loops adds ~80 nucleotides, reducing the amount of exogenous sequence within the study RNA. Like other RNA-binding protein-based tracking systems, λ_N-GFP binds its target RNA with high affinity and specificity; each λ_N repeat binds a single BoxB stem-loop with Kd = 22 nM (7). As with MS2-GFP and Bgl-mCherry, λ_N-GFP binding could influence the trafficking of the target RNA molecule. However, actin zipcode-containing mRNAs bound by λ_N-GFP are properly localized, providing evidence that λ_N-GFP does not alter trafficking of mRNAs to which it binds (26).

To visualize MMTV RNA trafficking in living cells, we created a subviral RNA (svRNA) construct containing the MMTV Ψ packaging sequence (87); the Rem response element, which is bound by the MMTV Rem protein to export full-length viral RNA from the nucleus (39, 60, 61); and four BoxB loops (Fig. 1A) (26). When expressed alone, λ_N-GFP was localized to the nucleus as expected (Fig. 1B, left) (26). When the svRNA was co-expressed with λ_N-GFP, there was no cytoplasmic GFP signal (Fig. 1B, middle), although co-expression of Rem, svRNA, and λ_N-GFP resulted in export of λ_N-GFP from the nucleus, confirming that Rem is required for the nuclear export of MMTV RNA (Fig. 1B, right) (61). The MMTV RNA appeared to accumulate in cytoplasmic granules, reminiscent of mRNA processing sites known as P-bodies (31). To address whether MMTV RNA was associated with RFP-Dcp1a, a component of P-bodies, living cells were imaged on a DeltaVision Elite microscope with a 60x 1.4 NA objective in a 37°C environmental chamber. Interestingly, a subset of MMTV RNA granules colocalized transiently with RFP-Dcp1a (Fig. 1C). Together, these data demonstrate that the λ_N-GFP system is a useful method for tracking retroviral RNAs in living cells.

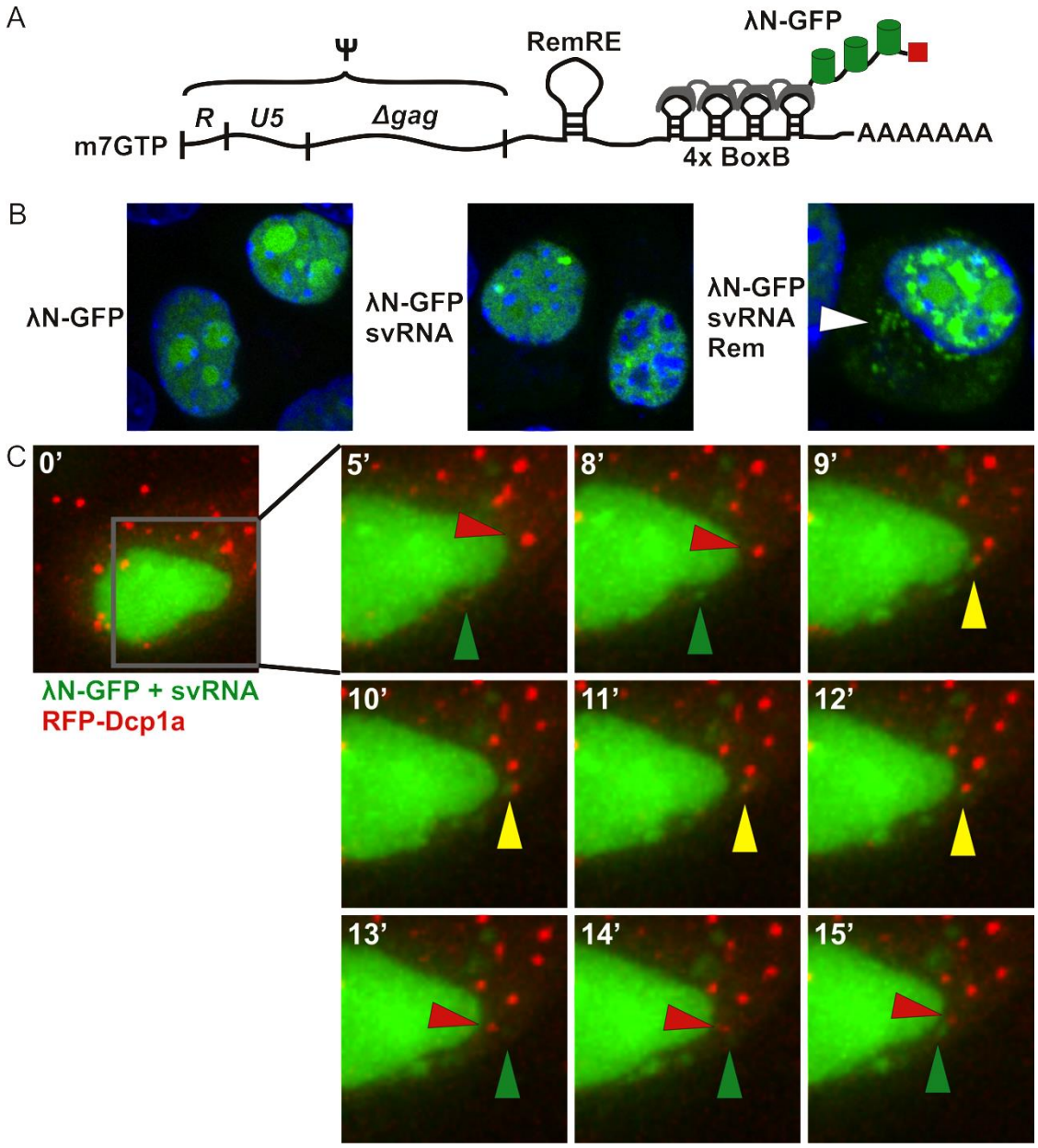


Figure 1. Localization of MMTV RNA with P-body proteins. **(A)** Diagram of MMTV subviral RNA containing the MMTV packaging signal (Ψ), the Rem response element (RemRE), and 4 BoxB loops bound by the λ_N-GFP reporter. **(B)** NMuMG cells transfected with λ_N-GFP alone (left); λ_N-GFP and svRNA (middle); or λ_N-GFP, svRNA, and Rem (right). Fixed cells were imaged using a Leica SP2 confocal microscope. White arrow indicates cytoplasmic accumulation of λ_N-GFP bound to svRNA. **(C)** Live-cell co-imaging of MMTV svRNA and RFP-Dcp1a in live NMuMG cells. Green arrows indicate svRNA alone; red arrows indicate RFP-Dcp1a alone; Yellow arrows indicate colocalization between the svRNA and RFP-Dcp1a.

3.4 PUM-HD

One of the major drawbacks to the RNA-binding protein-based systems discussed to this point is that all of these systems require the insertion of specific stem-loop structures into the RNA of interest. On the other hand, although endogenous transcripts can be visualized using the hybridization-based techniques discussed earlier, these techniques are technically challenging. These limitations have resulted in the development of a genetic probe based on the RNA-binding protein PUMILIO1 to track endogenous RNAs in living cells (77). The PUMILIO1 homology domain (PUM-HD) is composed of eight repeating units, each of which binds a single RNA nucleotide (102, 107). The wild-type PUM-HD recognizes the RNA sequence 5' UGUA(U/C)AUA, however PUM-HD can be engineered to recognize different sequences by mutating individual units based on a predictable set of rules (23, 56).

To visualize endogenous RNA molecules in living cells Ozawa *et al*. created two PUM-HD mutants that recognized adjacent sequences on the mitochondrial NADH dehydrogenase 6 RNA (77). The PUM-HD constructs were fused to the N- and C-terminal fragments of a split GFP reporter, so GFP fluorescence was only produced when both PUM-HD constructs were bound to the same RNA (Table 1) (77). Due to the high signal-to-noise ratio achieved through the split GFP reporter, this system has been used to visualize single, endogenous RNAs in living cells (104). The PUM-HD system has also been used to study the trafficking of tobacco mosaic virus RNA in living plant cells, and RNA localization appears to be unaffected by PUM-HD binding (97). As a result, the PUM-HD system may be a powerful technique to study trafficking of RNAs expressed from endogenous retroviruses and during authentic retroviral infection.

As with all RNA tracking systems, however, the PUM-HD system has some limitations. Background fluorescence may result from non-specific complementation of the split GFP fluorophore, reducing the signal-to-noise ratio (97). Because PUM-HD recognizes endogenous RNAs, it has the potential to bind transcripts non-specifically, much like an oligonucleotide probe. However, by recognizing two different eight nucleotide sequences in tandem, the PUM-HD system is theoretically able to distinguish its target from 4.3×10^9 different transcripts (77). However, the use of PUM-HD to study retroviral RNA trafficking may be complicated by the presence of endogenous retroviruses with high sequence homology to the exogenous virus of interest. Accordingly, PUM-HD probes may require greater optimization than other systems used to study retroviral RNA trafficking.

4. Direct RNA fluorescence

A common concern for all probe- or RNA binding protein-based RNA labeling systems is that binding of the reporter to the transcript of interest has the potential to alter trafficking of the transcript. Accordingly, an ideal system may be one where the RNA itself directly encodes a traceable marker. Recently, Paige *et al*. described a series of RNA aptamers called "Spinach" that fluoresce when bound to GFP-like fluorophores (Table 1) (78). The cell-permeable fluorophores are non-fluorescent in the absence of Spinach RNA and are non-toxic, making this system useful for live cell imaging (78). Furthermore, different combinations of RNA aptamers and fluorophores yield fluorescent RNAs with different excitation and emission spectra (78). Although the RNA-fluorophore interaction is highly specific (78), single molecule sensitivity has not been established in this system. Furthermore, while the Spinach system eliminates concerns regarding the influence of an oligonucleotide or protein reporter on RNA trafficking, the Spinach

aptamer itself could alter the trafficking of the transcript. However, Spinach-labeled 5S RNA appropriately relocalized from a diffuse cytoplasmic distribution to stress granules in 293T cells subjected to osmotic stress (78), suggesting that Spinach aptamers may not disrupt normal RNA trafficking or localization. Accordingly, while the Spinach system offers great promise as a novel system to visualize RNA transcripts in living cells, its utility for tracking single RNA molecules remains to be validated.

5. Conclusions

Technological and methodological advances have continued to improve the visualization of single RNA molecules in living cells. However, live cell RNA tracking studies are still not routine and technical challenges persist. For example, while hybridiziation-based approaches such as molecular beacons offer high sensitivity, delivery of the probe into cells can be difficult and requires careful optimization (8). On the other hand, RNA binding-protein based systems are plasmid-encoded, and therefore relatively easy to manipulate and deliver into cells by transfection. However, these approaches require the engineering of expression constructs and may introduce artifacts due to overexpression or mis-targeting of the transcript (81).

Clearly, not all RNA tracking systems are suitable to address every biological question. Molecular beacons and other hybridization-based approaches can be used to visualize endogenous transcripts, and because molecular beacons fluoresce only when bound to the target transcript, they can detect rapid changes in gene transcription (89). However, molecular beacons may not be well-suited for studying retroviral RNA trafficking because of high levels of sequence homology between endogenous and exogenous retroviruses. Although molecular beacons can be designed to detect single nucleotide differences between transcripts, this degree of specificity may not be ideal to visualize retroviral RNAs because of the high mutation rate associated with reverse transcription (86).

RNA binding protein-based approaches to live cell RNA tracking including MS2-GFP, Bgl-mCherry, and λ_N-GFP avoid potential pitfalls in differentiating between two closely related transcripts by binding to highly specific stem-loop structures. As a result, these systems can be used in combination to visualize two (or more) transcripts in the same cell. However, care must be taken to ensure that insertion of stem-loops into the target transcript does not affect RNA localization. Studies in yeast suggest that transcripts may tolerate insertion of stem-loops near the reading frame stop codon within the 3' untranslated region (37), although steps should be taken to empirically determine the effect of stem-loop insertion on RNA trafficking. A relative disadvantage of these approaches is that they cannot be used to visualize endogenous transcripts. As a result, the transcript of interest must be expressed from a plasmid and therefore may not be subjected to the same transcriptional or post-transcriptional regulation as its cellular counterpart. To circumvent this concern, endogenous transcripts in yeast and mice have been engineered to contain MS2 stem-loops (37, 54), although this approach is not practical for many applications.

Despite the limitations and caveats of RNA binding protein-based systems for RNA visualization, MS2-GFP and Bgl-mCherry have provided valuable insight into retroviral RNA trafficking, interactions with host factors, mechanisms underlying genome encapsidation, and the dynamics of particle assembly. Additionally, we have adopted the λ_N-GFP system to study MMTV RNA trafficking in living cells. We found that MMTV RNA interacts with cellular

RNA processing factors, including components of P-bodies. Proteins associated with P-bodies repress the translation of cellular mRNAs and target these transcripts for long-term storage or degradation (10). Additionally, P-bodies contain components of the RNA-interference pathway, as well as antiretroviral proteins including APOBEC3G (35). Accordingly, one function of P-bodies may be to restrict retroviral replication. However, proteins associated with P-bodies may also facilitate retroviral replication. For example, the P-body associated helicase Ddx3 is required for nuclear export of unspliced HIV-1 RNA (105). Furthermore, modulating expression of the P-body associated protein Mov10 interferes with HIV-1 replication (16, 32, 101), and the Gag proteins of HIV-1 and primate foamy virus-1 recruit Ago2 to the genomic RNA to facilitate viral replication (15). Our observation that MMTV RNA traffics to P-bodies adds support to the idea that diverse retroviruses interact with P-body proteins to facilitate viral replication. The data presented in this review highlight approaches used to reveal novel insights into retroviral biology by observing the dynamic trafficking and interactions of retroviral RNAs.

Progress Report for Aim 1b. Determine the role of PB/SG/RISCs in MMTV gRNA trafficking.

Publication: Bann DV, Beyer AR, Parent LJ. "A Murine Retrovirus Co-opts YB-1, a Translational Regulator and Stress Granule-associated Protein, to Facilitate Virus Assembly." J Virol. 2014 Feb 5. [Epub ahead of print] PMID: 24501406.

1. Abstract: The Gag protein of the murine retrovirus mouse mammary tumor virus (MMTV) orchestrates the assembly of immature virus particles in the cytoplasm, which are subsequently transported to the plasma membrane for release from the cell. The morphogenetic pathway of MMTV assembly is similar to yeast retrotransposons Ty1 and Ty3, which assemble virus-like particles (VLPs) in intracytoplasmic ribonucleoprotein (RNP) complexes. Assembly of Ty1 and Ty3 VLPs depends upon cellular mRNA processing factors, prompting us to examine whether MMTV utilizes a similar set of host proteins to facilitate viral capsid assembly. Our data revealed that MMTV Gag colocalized with YB-1, a translational regulator found in stress granules and P-bodies, in intracytoplasmic foci. The association of MMTV Gag and YB-1 in cytoplasmic granules was not disrupted by cyclohexamide treatment, suggesting these sites were not typical stress granules. However, the association of MMTV Gag and YB-1 was RNA dependent, and an MMTV RNA reporter construct colocalized with Gag and YB-1 in cytoplasmic RNP complexes. Knockdown of YB-1 resulted in a significant decrease in MMTV particle production, indicating that YB-1 plays a role in MMTV capsid formation. Live-cell imaging with fluorescence recovery after photobleaching (FRAP) analysis revealed that the population of Gag proteins localized within YB-1 complexes was relatively immobile, suggesting that Gag forms stable complexes in association with YB-1. Together, our data imply that the formation of intracytoplasmic Gag-RNA complexes is facilitated by YB-1, which promotes MMTV virus assembly.

2. Importance

Cellular mRNA processing factors regulate the post-transcriptional fates of mRNAs, affecting localization and utilization of mRNAs under normal conditions and in response to stress. RNA viruses such as retroviruses interact with cellular mRNA processing factors that accumulate in ribonucleoprotein complexes known as P bodies and stress granules. This report shows for the

first time that mouse mammary tumor virus, a mammalian retrovirus that assembles intracytoplasmic virus particles, commandeers the cellular factor YB-1, a key regulator of translation involved in the cellular stress response. YB-1 is essential for the efficient production of MMTV particles, a process directed by the viral Gag protein. We found that Gag and YB-1 localize together in cytoplasmic granules. Functional studies of Gag/YB-1 granules suggest they may be sites where virus particles assemble. These studies provide significant insights into the interplay between mRNA processing factors and retroviruses.

3. Results

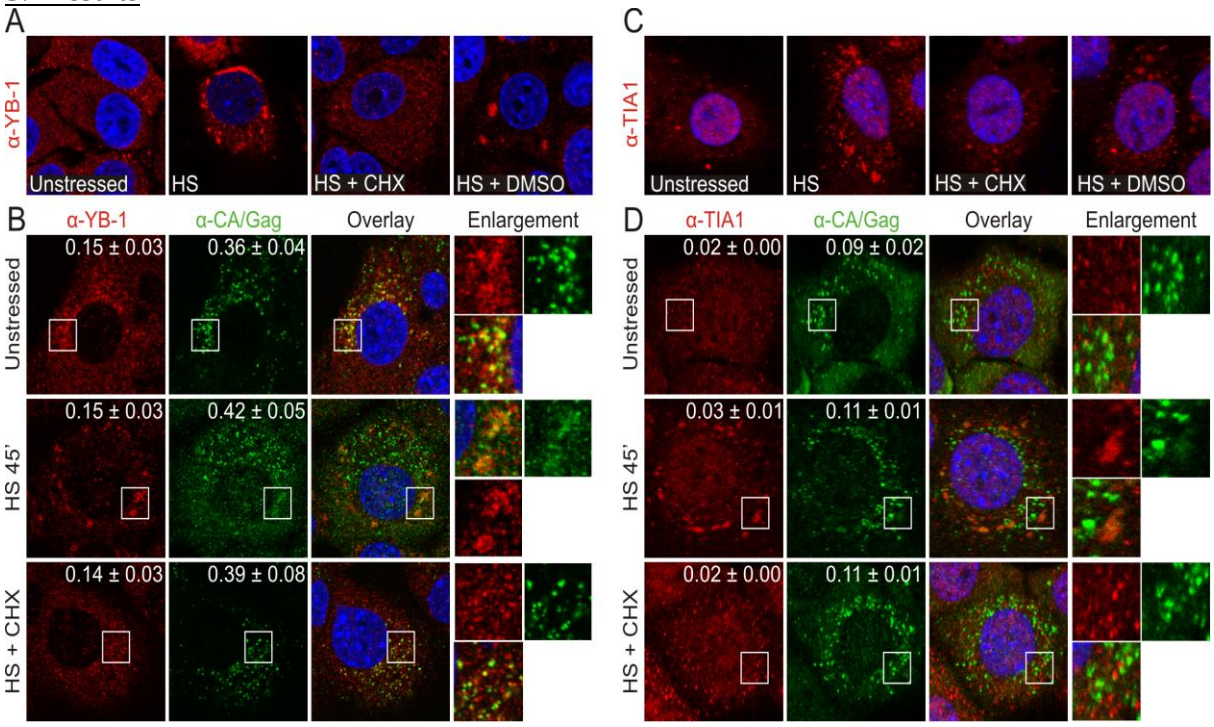


Figure 2. MMTV Gag colocalizes with endogenous YB-1. A) Uninfected NMuMG cells were unstressed, subjected to heat shock at 44°C for 45 minutes (HS), or heat shocked for 30 minutes at 44°C and treated with cyclohexamide (HS + CHX) or vehicle control (HS + DMSO) for 15 minutes at 44°C, then fixed and stained for YB-1. (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 YB-1 and Gag. Boxes indicate the area of enlargement. M_1 and M_2 coefficients are shown in the upper right-hand corner, and were calculated for ≥6 cells using Just Another Colocalization Plugin (JACoP) for ImageJ. Values are shown as the mean ± standard error of the mean. (D) MMTV(C3H) cells treated as above were immunostained for TIA1 and Gag. M_1 and M_2 values were calculated for 10 cells in each condition.

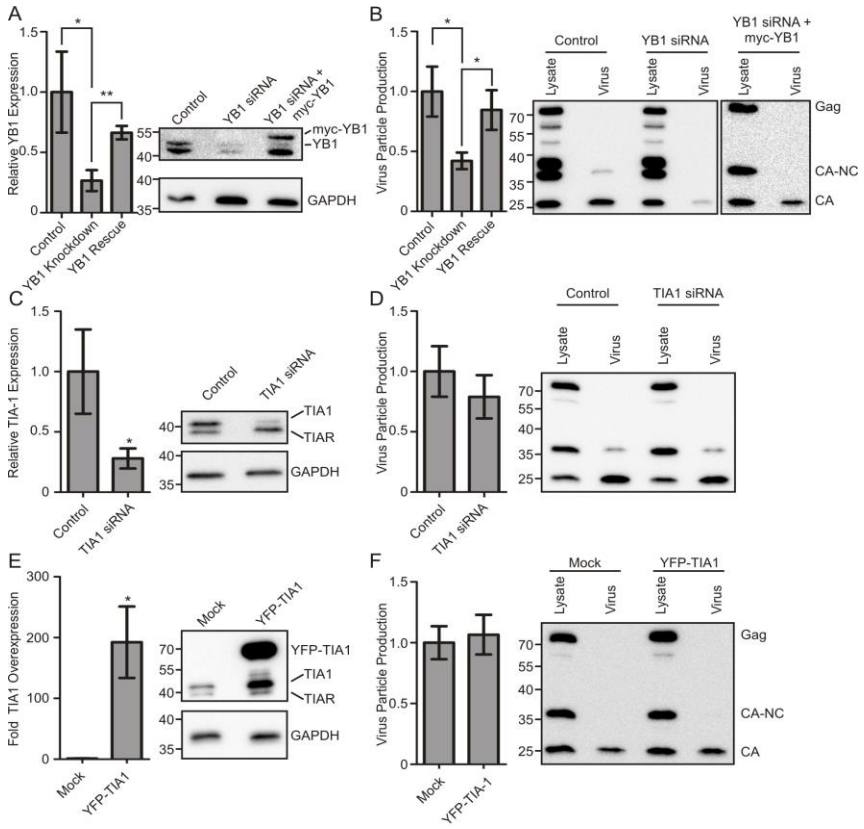


Figure 3. Functional role of YB-1 in MMTV assembly. (A) Graph showing mean YB-1 expression in NMuMG-C3H cells treated with YB-1 siRNA relative to cells treated with scrambled siRNA control. YB-1 expression was determined by Western blotting and standardized to GAPDH loading controls. A representative 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) (13). 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).

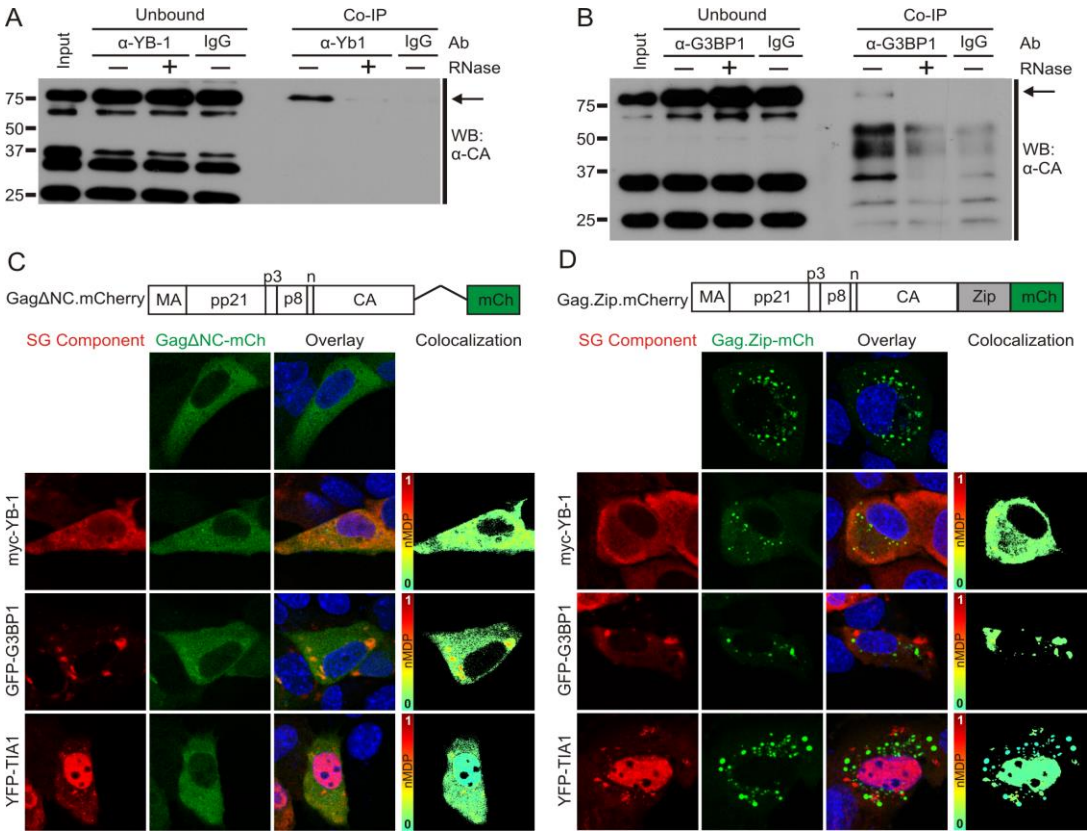


Figure 4. RNA-dependent interaction of Gag with SG proteins. Endogenous YB-1 (A) or G3BP1 (B) was immunoprecipitated from MMTV(C3H)-infected NMuMG whole cell lysates. Prior to the immunoprecipitation, some samples were treated with RNase A. Gag (arrow) was detected by Western blotting using mouse anti-CA antibody. (C) Diagram depicting the NC deletion mutant Gag.ΔNC-mCherry was expressed alone or co-expressed with myc-YB-1, GFP-G3BP1, or YFP-TIA1 in uninfected NMuMG cells. Colocalization colormaps were generated as above and are shown to the right. (D) Gag.Zip-mCherry, a mutant in which the NC domain of Gag has been replaced with the CREB1 leucine zipper domain, was expressed alone, or co-expressed with myc-YB-1, GFP-G3BP1, or YFP-TIA1 in NMuMG cells. Colocalization colormaps are shown to the right.

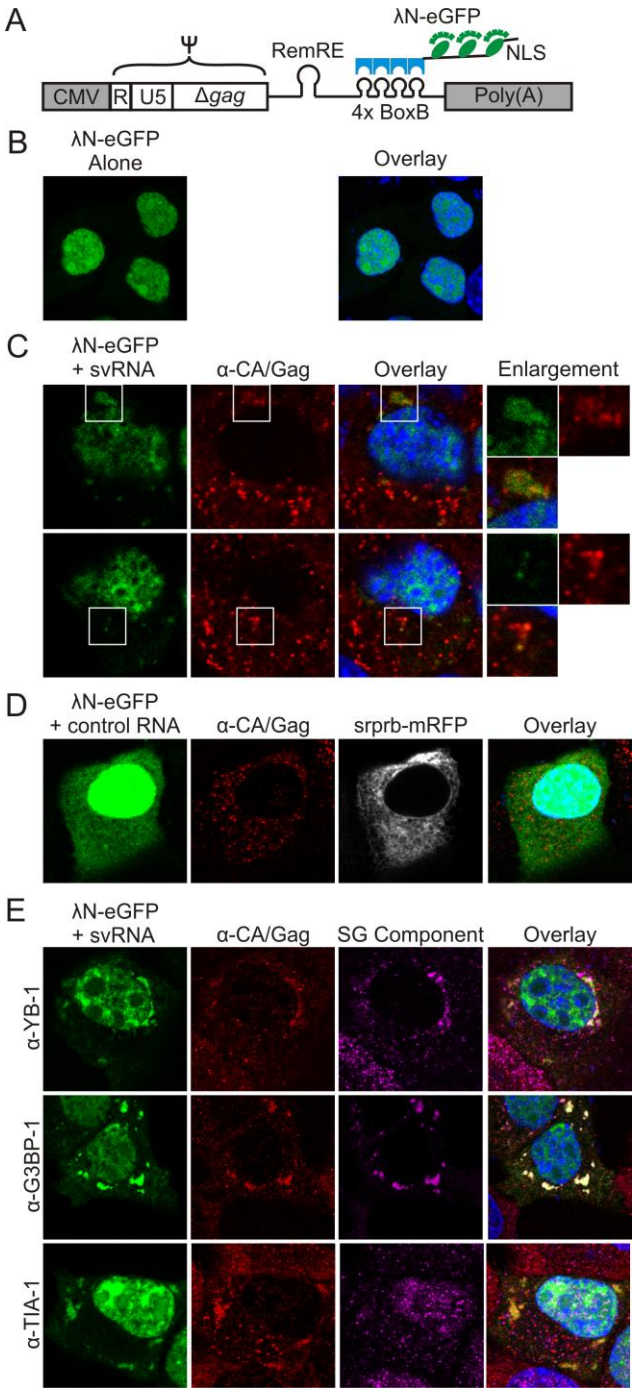


Figure 5. Localization of MMTV sub-viral RNA in infected cells. (A) Schematic diagram depicting the MMTV sub-viral RNA (svRNA) construct, which is driven by the CMV promoter and contains R, U5, the first 746 nucleotides of *gag* (Ψ); the Rem response element (RemRE); and four BoxB stem-loops, which are bound by the λ_N-eGFP reporter. The λ_N-eGFP reporter contains an M9 nuclear localization signal (NLS) (27). (B) Expression of the λ_N-eGFP reporter alone in MMTV-infected NMuMG.C3H cells. (C) Two planes through the same MMTV-infected NMuMG.C3H cell transfected with svRNA, λ_N-eGFP, and immunostained for Gag. The svRNA and Gag colocalize in cytoplasmic complexes. White boxes indicate the area shown

in “Enlargement.” (D) MMTV-infected NMuMG cells were transfected with λ_N-eGFP and immunostained for Gag and YB-1, G3BP-1, or TIA-1.

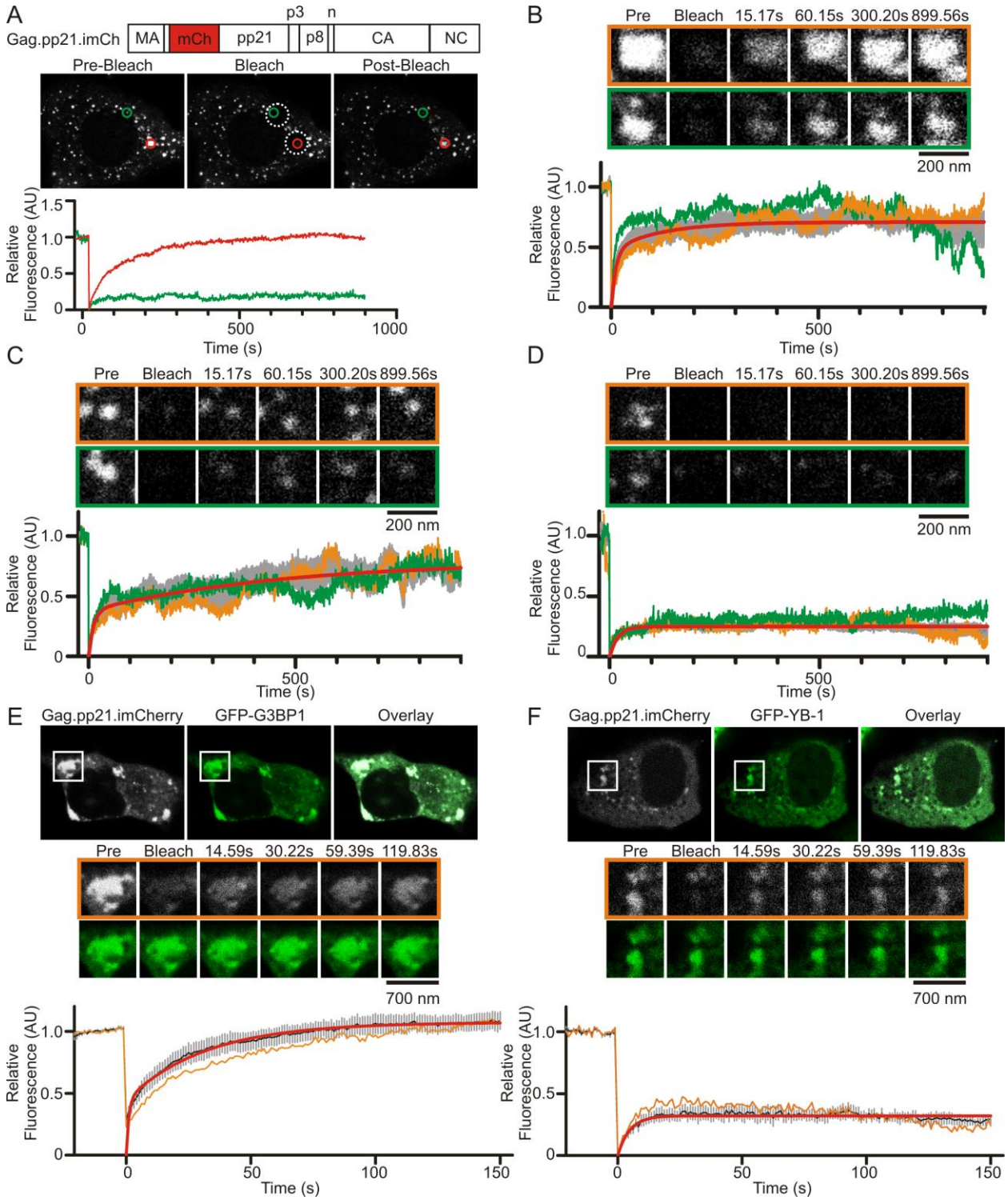


Figure 6. FRAP analysis of MMTV Gag in living MMTV.C3H-infected cells. (A) Parameters used to define different populations of MMTV Gag complexes in living C3H-infected NMumG cells. MMTV Gag was internally labeled with mCherry by inserting the fluorophore into the pp21 domain. The Gag.pp21.imCherry construct 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 discrete populations of foci with rapid (B), gradual (C), and limited (D) fluorescence recovery after photobleaching. Individual foci are shown in the orange and green boxes, with the relative fluorescence of each focus shown as the orange or green curve on the graph. The grey curves on each graph represent the mean fluorescence intensity from at least four foci ± standard error of the mean. The red line is the curve fit to the mean fluorescence intensity using GraphPad Prism using either a double (B, C) or single (D) exponential association curve. (E) FRAP analysis of Gag.pp21.imCherry within stress granules induced by expression of GFP-G3BP1 or GFP-YB-1 (F) in NMuMG-C3H cells. For each condition, a representative cell is shown at the initial pre-bleach time point, and a single cytoplasmic granule chosen for analysis is indicated by the white box. Enlarged images of the white boxed granule before (pre-bleach), during (bleach), and at various timepoints after photobleaching are shown for both the Gag.pp21.imCherry channel (grayscale, outlined by an orange line) and the GFP channel (green). Graphs show the mean fluorescence intensity for at least five granules (black line) with standard error of the mean (gray bars). The fluorescence intensiy of the representative focus is show as an orange line. Red lines are curves fit to the averaged data with double (E) or single (F) exponential association equations using GraphPad Prism. Time is indicated in seconds (s), with Pre indicating the Pre-bleached image.

4. Conclusions and Discussion:

Stress granules form in response to unfavorable cellular conditions, including viral infection. Although this stress response can create a hostile environment for pathogens, some viruses have evolved mechanisms to commandeer proteins involved in stress granule formation to promote virus replication (67). Here, we report that the betaretrovirus MMTV, unlike human retroviral pathogens HIV-1 and HTLV-I, does not disable the stress response by preventing stress granule formation. Instead, our data support the conclusion that MMTV Gag co-opts YB-1, a stress granule-associated protein and master regulator of translation, to facilitate virus assembly.

YB-1, a major component of cellular messenger ribonucleoprotein complexes (mRNPs), is present in both translating and non-translating mRNPs [(64, 65); reviewed in (57)]. In the cytosol, YB-1 is present in small cytoplasmic granules that coalesce into larger stress granules during unfavorable cellular conditions (24, 45). The mechanism by which YB-1 exerts post-transcriptional control is regulated by the amount of YB-1 that binds to an mRNA. At low YB-1:RNA mass ratios, the cold shock domain and C-terminal domain of YB-1 bind RNA, promoting an extended conformation of the mRNA, which favors translation (30, 95). Conversely, at high concentrations YB-1 multimerizes, compacting the mRNA and repressing translation (65, 95). In stress granules, YB-1 is thought to package translationally-repressed cellular mRNAs to prevent their degradation, allowing the mRNAs to return to polysomes for translation when conditions improve. In addition to regulating cellular translation, YB-1 modulates expression of some viral RNAs. For example, YB-1 represses Dengue virus replication by suppressing translation of viral RNA (80). YB-1 plays a different role in hepatitis C virus, where its recruitment to sites of virus assembly influences the balance between viral RNA replication and encapsidation (20). Recently, it was reported that YB-1 stabilizes

intracellular levels of HIV-1 and murine leukemia virus RNAs, resulting in an increase in virus particle production (52, 72).

In this report, we found that YB-1 colocalized with MMTV Gag in small cytoplasmic foci in unstressed cells. We speculate that YB-1 may associate with Gag and viral RNA in these granules and repress MMTV RNA translation, based on the failure of these granules to disassociate in the presence of cyclohexamide. Our reasoning is based on the assumption that MMTV Gag and YB-1 granules would disassemble with cyclohexamide treatment like "traditional" stress granules and P bodies if these granules contained RNAs that were recycling through polysomes. Further experiments will need to be done to directly test this idea. Alternatively, it is possible that MMTV Gag-YB-1 granules do not contain RNA. This explanation seems less likely given that MMTV Gag initiates immature capsid assembly in the cytoplasm in association with its RNA genome, and the data in Fig. 6 demonstrate that MMTV svRNA is present in Gag-YB-1 cytoplasmic granules.

The observation that MMTV Gag-YB-1 granules are constitutively present in infected cells is characteristic of GW or P bodies, which are present in unstressed cells and are involved in silencing, decay, transport, and stabilization of mRNAs (11, 44, 48, 55, 71, 82, 94, 96). However, Gag does accumulate in stress granules induced by heat shock and with overexpression of stress granule-associated proteins YB-1, TIA-1, G3BP1, TTP, HuR, and PABPc1. Nonetheless, it does not appear that the accumulation of Gag in stress granules plays an important role in particle assembly because knocking down or overexpressing TIA-1, a core stress granules component, did not have any effect on virus production. Furthermore, functional analysis of stress granules induced by G3BP-1 overexpression revealed that Gag was highly mobile in these complexes, arguing against stress granules as a site for Gag-driven immature capsid assembly. There is a close, dynamic interrelationship between stress granules and P bodies and many proteins, including YB-1, are constituents of both types of mRNA regulatory granules (5, 6, 43, 45-47, 70, 71). It is possible that the constitutive Gag-YB-1 granules contain components of P bodies, and this question is currently under study in our laboratory. The association of MMTV Gag with mRNA processing bodies (stress granules and P bodies) is complex and will require more detailed analysis to determine if there is a functional relationship between MMTV assembly and cellular factors in addition to the central role of YB-1 that was demonstrated in this manuscript. Based on our data, we propose that MMTV particles do not assemble in stress granules, nor is virus assembly inhibited by stress granule formation. Instead, our results favor the interpretation that MMTV may co-opt YB-1, and possibly other stress-granule and P body associated proteins, during the process of immature capsid formation.

Knockdown of YB-1 expression interfered with MMTV assembly, suggesting that YB-1 exerts a positive effect on virus particle formation, transport, or release. However, we did not observe a difference in Gag localization in cells expressing reduced levels of YB-1 (data not shown), implying that the defect did not alter the cytoplasmic localization of Gag but rather disrupted immature particle formation. The precise mechanism by which YB-1 facilitates MMTV particle formation is unclear, but our results have provided some clues. The interaction between Gag and YB-1 in MMTV-infected cells was RNAse-sensitive, suggesting that RNA bridges the interaction. The NC domain, which specifically binds to the viral genome, was required for localization of Gag to YB-1 granules, and dimerization of Gag through a nucleic acid

independent mechanism was not sufficient to mediate the Gag-YB-1 association. The MMTV subviral RNA colocalized with Gag and YB-1, suggesting that MMTV RNA is an integral part of Gag-YB-1 granules; however, one caveat to this interpretation is that the subviral RNA may not follow the same subcellular trafficking pathway taken by the full-length MMTV genomic RNA. Functional analysis of YB-1/Gag granules using FRAP revealed that these granules contained stable complexes of Gag that exchanged very little with free Gag in the cytoplasm. The best model fitting the kinetic data was a single exponential equation, indicating that Gag mobility was governed by a single binding event. Thus, the FRAP data define two functionally distinct populations cytoplasmic granules: (i) stress granules induced by G3BP1, which contain highly mobile Gag proteins and therefore do not likely represent sites of particle assembly; and (ii) Gag-YB-1 granules in which Gag proteins are relatively immobile, consistent with sites of immature capsid assembly.

Taken together, these data led us to construct a working model in which YB-1, and possibly other cellular proteins, associates with MMTV RNPs in cytoplasmic granules. We hypothesize that binding of YB-1 to viral RNA sequesters it away from translation machinery, essentially "marking" the viral RNA as a genome. The YB-1-viral RNP complex may serve as a landmark for Gag to identify sites to initiate capsid assembly. It is possible that Gag is recruited to YB-1 granules (through an unknown mechanism), displacing YB-1 from the viral RNA due to high affinity binding between Gag and the genomic RNA psi-packaging signal. YB-1 also binds cellular mRNAs in small cytoplasmic granules, so in the absence of viral RNA, MMTV, like other retroviruses, may encapsidate cellular RNAs. Alternatively, it is possible that Gag and YB-1 associate first, and the viral genomic RNA is recruited independently to Gag-YB-1 granules. Further experimentation is needed to refine our understanding of the mechanism by which YB-1 promotes Gag-mediated assembly of MMTV.

The recruitment of Gag to YB-1 granules where genomic viral RNA may be sequestered would represent a mechanism to circumvent cellular antiviral defenses. This strategy may be common among other retroviruses and 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 (29). These LINE-1 RNPs contain LINE-1 RNA and the retrotransposon proteins Orf1p and Orf2p, as well as YB-1 and other stress granule-associated proteins (29, 36). Additionally, YB-1 has a stabilizing effect on the genomic RNA of HIV-1 and psi-containing RNAs of MLV-based vectors, and YB-1 promotes HIV and MLV particle production (51, 72). Considering these data together, we propose that targeting mammalian retroviral and retrotransposon structural proteins to YB-1 containing cytoplasmic granules where viral RNAs are sequestered may be a mechanism to counteract cellular defenses and promote replication of retroelements and retroviruses. Importantly, these findings are similar to data from HIV-1 and Ty3 indicating that assembly of retrovirus or retrotransposon particles occurs in conjunction with stress granule or P body-associated proteins, but not in stress granules or P bodies themselves (2, 85, 88).

Progress Report for Aim 1c. Determine whether the mi-RNA pathway influences MMTV gRNA localization.

To address this question, we first characterized the subcellular localization of MMTV RNA and its association with host proteins. We used the λN-eGFP reporter to label the MMTV-derived RNA construct described in Aims 1a and 1b to visualize the localization of the subviral RNA (svRNA) as a surrogate for full-length MMTV RNA (see Figure 5, above).

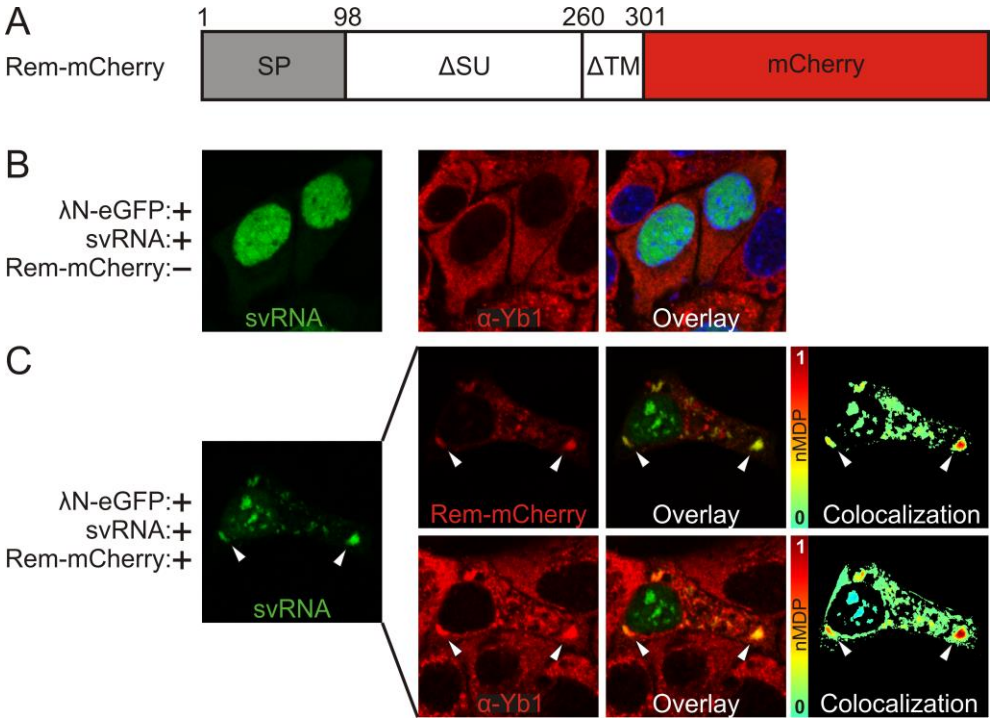


Figure 7. MMTV subviral RNA colocalizes with the MMTV Rem protein and the cellular factor YB-1 in the cytoplasm. MMTV Rem is required for the export of unspliced viral RNA from the nucleus and Rem remains colocalized with viral RNA in the cytoplasm. (A) Schematic diagram showing the domains of MMTV Rem protein with SP (signal peptide), partial deletion of surface (SU) and TM (transmembrane) domains, and C-terminal fusion of the mCherry protein. (B) Expression of λN-eGFP reporter construct is confined to the nucleus due to the insertion of a nuclear localization signal (left panel) and the svRNA remains in the nucleus. Cells were immunostained for endogenous YB-1 (CY3, red; middle panel). Overlay of GFP, Cy3, and DAPI channels are shown in the right image. (C) Single left panel: Expression of MMTV Rem-mCherry with svRNA induced the export of svRNA from the nucleus. Note the presence of svRNA in the cytoplasm, indicated by white arrowheads. Top set of panels: Rem-mCherry (red) remains associated with svRNA (green) as demonstrated by yellow color in the Overlay. Colocalization colormapping (far right image) shows colocalization as a color gradient, with highest levels of colocalization as red and lower levels as green. The svRNA-Rem foci are red, indicating a high intensity of colocalization. Lower set of images: The same cells as shown in the upper panel were immunostained with anti-YB1 antibody to detect endogenous YB-1 (red). The overlay and colocalization colormapping indicate that YB-1 accumulates with Rem and svRNA in discrete cytoplasmic foci.

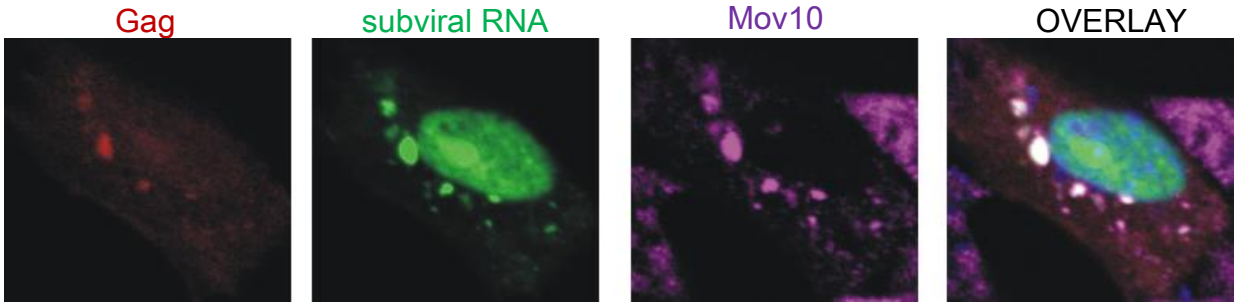


Figure 8. The P body and RISC (RNA-induced silencing complex) protein Mov10 colocalizes with MMTV Gag and svRNA in cytoplasmic complexes. Confocal images were obtained through the center of the cell demonstrating that Gag, svRNA, and Mov10 colocalize in the cytoplasm. Mov10 plays an important role in the replication of several retroviruses (e.g., HIV-1 and murine leukemia virus) and endogenous retroelements (LINE-1).

Progress Report for Aim 2a. Develop methods for detecting endogenous LINE-1 activity

We obtained a plasmid expressing the murine LINE-1 element and a LINE-1 mutant that is defective in retrotransposition. We have been testing conditions to detect retrotransposon activity in a number of different cell types, including HeLa, U2OS, and NMuMG cells. We have encountered difficulties with the detection of LINE-1 activity, as the cells have not tolerated expression of LINE-1. We are continuing to troubleshoot the assay by titrating the concentration of the plasmid, trying different transfection reagents, and using various drug concentrations for selection of LINE-1 expressing cells. In addition, we will continue to develop a PCR-based assay to detect endogenous LINE-1 RNA levels.

We have also cloned LINE-1 Orf1 protein (Orf1p) fused to mCherry to study the localization of Orf1p in MMTV-infected cells. Out preliminary data indicate that MMTVsvRNA colocalizes with LINE-1 Orf1p. Further experiments are underway to look for colocalization of MMTV Gag, svRNA, and LINE-1 Orf1p.

Progress Report for Aim 2b. Develop a packaging assay to quantitate MMTV genomic RNA in virions.

To detect MMTV RNA in virus particles, we have developed a reverse-transcriptase (RT) PCR-based assay. Virus particles are collected in the supernatant of infected cells, pelleted through a sucrose cushion, and viral RNA is extracted. The RNA from virions is subjected to RT-PCR and run on an agarose gel. Using this method, we can detect MMTV viral RNA. We have decided not to pursue a ribonuclease-protection assay because we have had initial success with the RT-PCR packaging assay. For most purposes, detecting viral RNA using a semi-quantitative approach will be adequate, in which we will perform dilutions of the viral RNA template prior to RT-PCR. If needed, we will perform quantitative RT-PCR using an instrument available in our core facility.

Progress Report for Aim 2c. Determine whether components of RNA silencing complexes (RISCs) inhibit LINE-1 retrotransposition and/or MMTV replication.

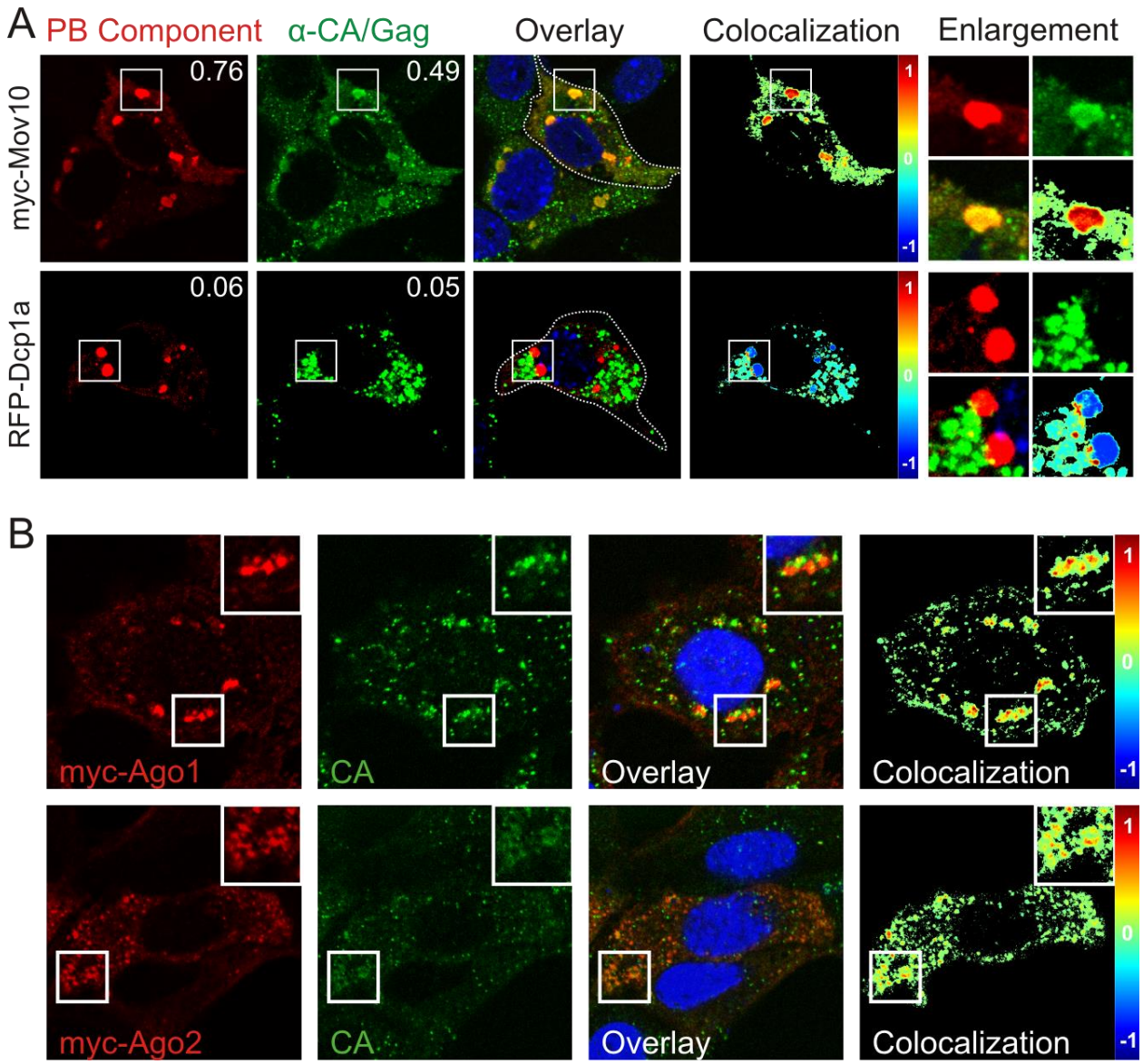


Figure 9. The MMTV Gag protein colocalizes with components of cellular RISC machinery. (A) MMTV-infected cells were transfected with myc-tagged Mov10 or RFP-tagged DCP1a (decapping enzyme 1a) and immunostained for Gag using a anti-CA antibody/ The overlay images and colocalization colormapping show that myc-Mov10 colocalizes strongly with Gag whereas RFP-Dcp1a appear to be adjacent to gag cytoplasmic foci. (B) RISC components Ago1 and Ago2 (Argonaute proteins) were expressed in MMTV-infected cells and detected using anti-myc antibodies. The images demonstrate that both Ago1 and Ago2 colocalize with MMTV Gag in cytoplasmic aggregates. The functional relevance of these interactions is being tested.

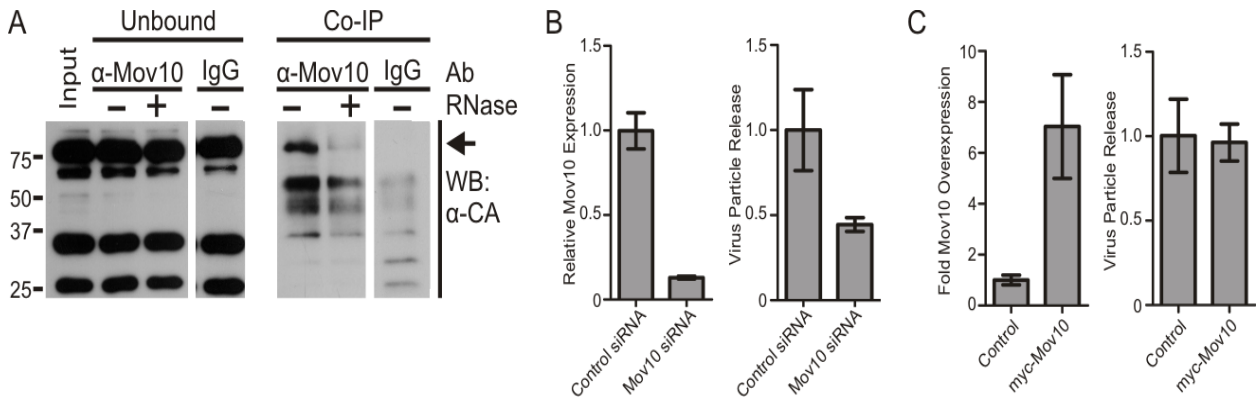


Figure 10. MMTV Gag interacts with RISC protein Mov10, which is required for efficient virus particle production. (A) Co-immunoprecipitation was performed in MMTV-infected cells using anti-Mov10 antibody to pull down endogenous Mov10. Proteins were transferred to a membrane and Western blotting was performed using anti-CA antibody to detect Gag. Gag was co-immunoprecipated with Mov10 in the absence of RNAse, but the interaction was disrupted when the cell lysates were pre-treated with RNAse. This result indicates that Gag-Mov10 interaction is RNA dependent. (B) MMTV-infected cells were transfected with an si-RNA specific for Mov10 or with a nonspecific control siRNA. Mov10 expression was significantly reduced by the anti-Mov10 siRNA (~15% of baseline levels). In the same cells, cell culture supernatants were collected and virus particles were isolated. Detection of viral proteins was performed using Western blotting with anti-CA antibody. The ratio of viral proteins present in the supernatant was compared to the amount of viral gag protein expressed in the cells to yield the virus particle release. In the Mov10 knockdown cells, MMTV particle release was reduced by approximately 50%, indicating that Mov10 plays a role in MMTV particle assembly. (C) The effect of Mov10 overexpression on MMTV particle production was tested. In the left panel, the Mov10 protein was expressed ~7-fold higher in the myc-Mov10 transfected cells. In the right panel, the data show that there was no change in the efficiency of virus particle production with or without myc-Mov10 overexpression. We plan to test the effects of Mov10 knockdown on murine LINE-1 expression once the retrotransposition assay has been optimized. We will also examine whether the localization of MMTV Gag and LINE-1 Orf1p is altered in the Mov10 knockdown cells.

Publications:

1. Bann DV and Parent LJ, "Tracking Retroviral RNAs in Living Cells," *Viruses.* 2012 Jun;4(6):963-79. doi: 10.3390/v4060963. Epub 2012 Jun 8. PMID: 22816035.

2. Stake MS, Bann DV, Kaddis RJ, Parent LJ. "Nuclear trafficking of retroviral RNAs and Gag proteins during late steps of replication." Viruses. 2013 Nov 18;5(11):2767-95. doi: 10.3390/v5112767. PMID: 24253283.

3. Bann DV, Beyer AR, Parent LJ. "A Murine Retrovirus Co-opts YB-1, a Translational Regulator and Stress Granule-associated Protein, to Facilitate Virus Assembly." J Virol. 2014 Feb 5. [Epub ahead of print] PMID: 24501406.

Abstracts presented at national/international meetings:

1. Bann DV, Beyer AR, and Parent LJ. Y-box-binding protein-1 (YB1) regulates mouse mammary tumor virus assembly, 24th Workshop on Retroviral Pathogenesis, Philadelphia, PA, October 24-27, 2012

2. Bann DV, Beyer AR, and Parent LJ. Stress Granule Protein YB-1 is Required for Assembly of the Betaretrovirus Mouse Mammary Tumor Virus, 112th General Meeting of the American Society for Microbiology, San Francisco, CA, June 16 – 19, 2012

3. Bann DV, Beyer AR, and Parent LJ. Stress granule protein YB-1 is required for assembly of the betaretrovirus mouse mammary tumor virus. National MD/PhD Student conference. July 6 - 8, 2012 Keystone, CO

4. Bann DV, Beyer AR, and Parent LJ Intracellular Trafficking of MMTV Gag and RNA during Late Replication, NIH Think Tank, Frederick, MD, April 29, 2013

5. Parent LJ. Retrovirus-Cell Interactions: Hijacking Nuclear Machinery for Virus Replication 113th General Meeting of the American Society for Microbiology, May 18-21, 2013

6. Bann DV, Beyer AR, and Parent LJ. The mRNA-binding protein YB-1 participates in the assembly of a betaretrovirus, Structural Biology Related to HIV/AIDS, NIH Campus, Bethesda, Maryland, June 20-21, 2013.

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18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04),

the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Tracking Retroviral RNAs in Living Cells	Bann DV and Parent LJ	Viruses	May 2012	☐Submitted ☐Accepted X Published
2. Nuclear trafficking of retroviral RNAs and Gag proteins during late steps of replication	Stake MS, Bann DV, Kaddis RJ, Parent LJ	Viruses	October 2013	☐Submitted ☐Accepted X Published
3. Nucleolar Trafficking of the Mouse Mammary Tumor Virus Gag Protein Induced by Interaction with Ribosomal Protein L9	Beyer AR, Bann DV, Rice B, Pultz IS, Kane M, Goff SP, Golovkina TV, Parent LJ	Journal of Virology	Sept 2012	☐Submitted ☐Accepted X Published
4. A Murine Retrovirus Co-opts YB-1, a Translational Regulator and Stress Granule-associated Protein, to Facilitate Virus Assembly	Bann DV, Beyer AR, Parent LJ	Journal of Virology	December 2013	☐Submitted X Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

A manuscript describing the role of Mov10 in MMTV particle assembly is in preparation. We plan to submit this manuscript to the Journal of Virology in the next 3-4 months.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Leslie J. Parent
Professor, Departments of Medicine and Microbiology& Immunology
Chief of the Division of Infectious Diseases and Epidemiology

Education/Training:
Dartmouth College, Hanover, NH A.B. 06/83 Psychology/Biology
Duke University School of Medicine, Durham, NC M.D. 05/87 Medicine
Penn State College of Medicine, Hershey, PA Fellowship 06/94 Infectious Diseases
Penn State College of Medicine Fellowship 06/97 Retrovirology

Research Interests: As a physician-scientist, I became interested in retrovirology through my experiences with patients infected with HIV, which was discovered when I was in medical school. My commitment to basic biomedical research was cemented during the year I spent in in the HHMI/NIH Research Scholars Program at the NIH where I worked in the laboratory of Dinah Singer, Ph.D. in molecular biology and immunology. My passion to understand the molecular mechanisms of retroviral replication was further developed during my research intensive residency with Warner Greene, M.D., Ph.D. (Duke University Medical School) and my postdoctoral training with John Wills, Ph.D. (Penn State College of Medicine) while I was a fellow in Infectious Diseases.

Since becoming an independent scientist, I have received continuous NIH funding for my work on retrovirus assembly using a comparative retrovirology approach to define unique and common features of Rous sarcoma virus (RSV), human immunodeficiency virus type 1 (HIV-1), and mouse mammary tumor virus (MMTV). Members of my laboratory discovered that the RSV Gag protein undergoes nucleocytoplasmic trafficking, which is required for the efficient encapsidation of viral genomic RNA into virions. We mapped the nuclear import and export signals in RSV Gag, identified cellular co-factors that mediate Gag transport, and determined the mechanism that controls spatiotemporal nucleocytoplasmic trafficking of Gag. We found that the Gag proteins of HIV-1 and MMTV also undergo nuclear localization. We have recently developed methods to study Gag-RNA trafficking in living cells. We have characterized an extensive set of Gag mutants, which we leverage in our mechanistic and structural studies. The long-term aim of these studies is to contribute to the development of inhibitors targeting the late stages of the retrovirus replication cycle.

Positions and Employment:
1987-1991 Resident in Internal Medicine, Duke University Medical Center, Durham, NC
1989-1991 Clinical Investigator Pathway in Internal Medicine, Duke University Medical Center, Durham, NC
1991-1994 Fellow, Infectious Diseases, Hershey Med Center/Penn State College of Medicine, Hershey, PA
1992-1997 Postdoctoral Fellow, Medicine and Microbiology & Immunology, Penn State College of Medicine
1994-1995 Instructor, Div. of Infectious Diseases, Department of Medicine, Penn State College of Medicine
1995-2003 Assistant Professor of Medicine, Penn State College of Medicine
1997-2003 Assistant Professor of Microbiology & Immunology, Graduate Faculty, Molecular

Medicine/Integrated Biosciences Faculty, Cell and Molecular Biology Program Faculty, MD/PhD Faculty and Steering Committee, Penn State College of Medicine
2003-2005 Associate Professor of Medicine, Microbiology & Immunology, Penn State College of Medicine
2005- Tenured Professor of Medicine, Microbiology & Immunology, Penn State College of Medicine
2007- Chief, Division of Infectious Diseases and Epidemiology, Penn State College of Medicine

Professional Experience and Memberships:
1985-1986 HHMI/NIH Research Scholar, laboratory of Dinah Singer, Ph.D., NCI/NIH, Bethesda, MD
1989-1991 Clinical Investigator, laboratory of Warner Greene, M.D., Ph.D., HHMI, Duke University Medical Center, Durham, NC
1992-1997 Postdoctoral Fellow, laboratory of John Wills, Ph.D., Department of Microbiology & Immunology, Penn State College of Medicine; studied molecular mechanisms of retrovirus assembly
2001-02 Co-Chair, Assembly Session, Cold Spring Harbor Retroviruses Meeting
2003 *Ad hoc* member, Virology Study Section, National Institutes of Health; Ad hoc reviewer AAAS
1999-present Reviewer for *PNAS, J. Virol., J. Mol.Biol., Clin.Inf.Dis., Eukaryotic Cell, Nat. Struc .Mol. Biol., PLOS One, Virology, Retrovirology*
2004-2008 Virology A (VIRA) Study Section, National Institutes of Health, Standing member
2005-present Editorial Board, *Journal of Virology;* Member, American Society for Virology
2011- Chair, Promotion and Tenure Committee, Penn State College of Medicine
2011- Co-Director, MD/PhD Program, Penn State College of Medicine
2011- Co-Director, Pilot and Collaborative Translational and Clinical Studies, NIH Clinical and Translational Studies Award, Penn State Clinical and Translational Studies Institute
2011 Co-Chair, Assembly Session, Cold Spring Harbor Retroviruses Meeting
2012 Co-Organizer, 24th International Workshop on Retroviral Pathogenesis, Philadelphia PA
2013 Chair, Division T (RNA Viruses), American Society for Microbiology
2013 Session Convener, NCI-supported "Workshop on Post-transcriptional Regulation of Virus Expression," Syria, Virginia, July 18-20
2013 Session Chair, "Zoonosis, Assembly and Accessory Proteins," 25th International Workshop on Retroviral Pathogenesis, Reykjavik, Iceland, August 7-10
2013 *Ad hoc* member, NIH/NIAID Study Section, AIDS Research Review Committee (AIDSRRC) for career development awards and institutional training grants, December 11, 2013