Georgia Journal of Science

Volume 75 *No. 2 Scholarly Contributions from the Membership and Others*

Article 15

2017

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Cover Page Footnote
We would like to thank Dr. M.K. Kandasamy and the University of Georgia Biomedical Microscopy Core for the training and use of their confocal microscope, Dr. Gary Ketner at Johns Hopkins University for viruses and cell lines, Dr. Patrick Hearing at Stony Brook University for cell lines, and the Georgia College Department of Biological and Environmental Sciences for their continued support of our research.

EFFECTS OF ADENOVIRUS INFECTION ON THE LOCALIZATION OF CELLULAR PROTEIN Pat1b

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ABSTRACT

Adenoviruses are a diverse family of nonenveloped, double-stranded DNA viruses with a variety of vertebrate hosts including humans. Over 50 serotypes of human adenovirus have been identified, and cause a number of illnesses, including conjunctivitis, gastroenteritis, and respiratory infections. The life cycle of adenovirus is divided into immediate early, early, and late phases, with immediate early proteins controlling transcription and the cell cycle, early proteins being largely regulatory, and late proteins being structural. Early proteins such as E4 11k have been demonstrated to relocalize key cellular proteins, including proteins found within mRNA processing bodies (p-bodies). It is hypothesized that E4 11k may affect gene expression during the late phase by disrupting p-bodies. One major p-body protein is the scaffolding protein Pat1b (the corresponding gene being PATL1). Pat1b is known to play a role in transcriptional regulation; however, research concerning its localization during an adenovirus infection had not been observed. Our preliminary results show that cytoplasmic Pat1b foci increase in number during an adenovirus infection. Additionally, we consistently found large nuclear aggregates of Pat1b in mock- and adenovirus-infected cells.

***Keywords**:* adenovirus, Pat1b, p-bodies

INTRODUCTION

Adenoviruses are nonenveloped, icosahedral viruses with linear, double-stranded DNA genomes. Also known to infect animal populations, adenovirus has been found to cause a diverse array of illnesses in humans. Adenovirus-associated illnesses range from mild respiratory infections in young children to life-threatening, multiorgan disease in immunocompromised patients. Transmission of adenovirus occurs in a variety of ways, from direct contact with the conjunctiva to inhalation of aerosolized particles, fecal-oral routes, and exposure to infected tissue and bodily fluids. While a live oral vaccine has been developed for adenovirus serotypes 4 and 7, it is approved only for military personnel ages 17 to 50 (Choudhry et al. 2016). The serotype commonly used in laboratory settings as well as in this paper is adenovirus serotype 5 (Ad5).

The adenovirus life cycle can be divided into three phases based on its replication process: an immediate early phase, an early phase, and a late phase. E1A, the first viral gene to be expressed in the immediate early phase, has been shown to stimulate transcription of a number of cellular genes (Berk 1986), as well as all other early viral genes (Grand 1987). E1A is also known to influence the cell cycle by inducing the

activation of the G1/S-phase checkpoint (Nemajerova et al. 2008) and arresting cells in S phase (Grand et al. 1998). Early genes, transcribed during the early phase, are predominantly regulatory proteins that target a variety of cellular activities, including the cell cycle and antiviral responses, and establish an intracellular environment that is favorable for viral replication. Viral DNA replication indicates the transition from early to late phase. In the late phase, there is an overall decrease in cellular protein synthesis. The late genes are largely structural, encoding the proteins used to build progeny virus for future infections, as well as proteins required for maturation and assembly of virions.

Studies of adenovirus with mutations in early region 4 (E4), in particular, E4 11k and E4 34k, have revealed that it is necessary for efficient viral replication and late gene expression (Halbert et al. 1985). E4 34k functions in concert with another early protein, E1B 55k, to form an E3 ubiquitin ligase complex (Harada et al. 2002) and this complex has some redundant functions with E4 11k. Double mutants of either E1B 55k/E4 11k or E4 34k/E4 11k show a severe replication defect while single mutants of any of the three have a more mild effect (Bridge and Ketner 1989; Huang and Hearing 1989). The E4 11k protein (encoded by the E4 ORF3 gene) and E1B 55k have been shown to participate in the shutoff of host protein synthesis. HeLa cells infected with E1B 55k/E4 11k double mutant virus display reduced levels of late viral proteins and increased levels of cellular proteins; conversely, normal expression of E1B 55k and E4 11k in HeLa cells results in increased expression of late viral proteins and a distinct reduction in the expression of cellular proteins (Shepard and Ornelles 2004). While the deletion of E4 ORF3 alone has little effect on viral replication in wild-type adenovirus infections, its presence has been shown to enhance viral replication in the absence of E1B 55k (Shepard and Ornelles 2004). In HeLa cells infected with E1B 55k/E4 11k double mutant virus, concatemeric viral DNA is produced, and late viral gene expression is significantly reduced.

Both E1B 55k and E4 11k have been shown to enhance viral replication by inhibition of the double-strand break repair pathway, which usually functions to repair cellular DNA damage. In E1B 55k/E4 11k double mutant virus, cellular repair proteins such as the Mre11-Rad50-Nbs1 (MRN) complex accumulate at viral replication centers during adenovirus infection (Evans and Hearing 2005), resulting in ligation of linear viral genomes into concatemers (Boyer et al. 1999). The reorganization of the MRN complex by E4 11k has been shown to be imperative for viral replication in the absence of E1B 55k or E4 34k (Evans and Hearing 2005). Similarly, the assembly of E1B 55k and E4 34k has been shown to induce the degradation of Mre11 (Stracker et al. 2002), DNA ligase IV (Baker et al. 2007), and Bloom helicase (Orazio et al. 2010), other cellular proteins that are necessary for double-strand break repair.

E4 11k has also been shown to facilitate viral replication by disrupting antiviral responses and relocalizing key cellular proteins found within mRNA processing bodies (p-bodies). P-bodies are cytoplasmic aggregates of mRNAs and proteins, many of which are enzymes required for mRNA decay (Kulkarni et al. 2010). Research has also indicated that p-bodies play a role in miRNA-induced mRNA silencing, and that some mRNAs may be stored in p-bodies for later translation (Brengues et al. 2005). During adenovirus infection, several p-body proteins have been shown to be relocalized to aggregates of misfolded proteins known as aggresomes, which colocalize with γ-tubulin and are believed to function as sites for rapid degradation of proteins (Weitzman and Ornelles 2005.) While aggresome formation can be induced by stressors such as chemical treatment and viral infection, the localization of the p-body protein, Ddx6, to aggresomes

has been demonstrated to occur specifically as a result of Ad5 E4 11k expression during adenovirus infection (Greer et al. 2011). Additional p-body proteins that are relocalized after E4 11k expression include Lsm1 and Ge1.

Ddx6, a cellular RNA helicase, is known to function in both translational suppression and mRNA decay, and has been shown to work with Pat1b to posttranscriptionally control gene expression by promoting the decapping of mRNA. Furthermore, the binding of Ddx6 to Pat1b has been identified as a key step in the formation of p-bodies (Ozgur and Stoecklin 2013), and Pat1b can form p-bodies in the absence of binding to Ddx6. In HeLa cells, the localization of Pat1b has been observed under various conditions. When the CRM1 (XPO1) pathway is inhibited by leptomycin B (LMB), it has been shown to be nuclear, sometimes colocalizing with SC35, a splicing factor, in nuclear speckles, and other times with promyelocytic leukemia protein (PML) nuclear bodies. When transcription is inhibited by actinomycin D, Pat1b has be found in nucleolar caps. Given the role of PML bodies in both transcription and posttranslational modifications, it has been suggested that Pat1b may also be linked to transcription, in addition to splicing and ubiquitination (Marnef et. al 2012).

As the relocalization of a variety of cellular proteins during viral infection, specifically p-body proteins, has been repeatedly demonstrated, we sought to identify the pattern of localization of Pat1b during adenovirus infection. We hypothesized that its relocalization, if demonstrated, would also be an E4 11k-dependent mechanism possibly related to the control of cellular gene expression. However, since Pat1b can form cytoplasmic foci in the absence of Ddx6, we acknowledged that if E4 11k is only interacting with Ddx6, Pat1b may remain in cytoplasmic foci. Additionally, we aimed to determine the localization of Pat1b in relation to that of other p-body proteins, namely Ddx6.

MATERIALS & METHODS

Cell Culture, Viruses, and Infections

A549 cells (adenocarcinomic human alveolar basal epithelial cells) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin, and incubated at 37 ˚C in 5% CO_2. Viruses were purified on CsCl equilibrium gradients. The viruses used include wild-type adenovirus serotype 5 (Ad5) and the Ad5 E1-replacement HA-E4ORF3 virus (previously described by Evans and Hearing 2003). Cells were seeded on 12 mm collagen-coated coverslips (neuVitro Corporation) in a 24-well plate and infected with wild-type and mutant viruses at an MOI of 10. Viral dilutions were prepared in DMEM at a volume of 300 µL per well and incubated for 20 hours.

Transfections

Some cells were transfected with the plasmid expressing RFP-tagged Ddx6 (Minshall et al. 2009) either alone or 4 hours prior to infection. Cells were transfected using polyethylenimine (PEI). First, 0.5 µg of DNA was added to 30 µl of OPTI-MEM (reduced serum medium without phenol red). Next, 1.5 µg of PEI was added to the DNA mixture. The solution was immediately vortexed and incubated at room temperature for 30 min. Finally, it was added to the well and incubated at 37 ˚C.

Antibodies

For immunofluorescence, antibodies were prepared in 10% goat serum in PBS. The antibodies used for immunofluorescence were as follows: anti-Ddx6 polyclonal antibody (Novus Biologicals) at 1:3000; anti-HA monoclonal antibody (clone 16B12, BioLegend) at 1:2000; anti-Pat1b polyclonal antibody (Novus Biologicals) at 1:100; and anti-DBP monoclonal antibody (clone B6-8) at 1:100. The secondary antibodies used were Alexafluor 488 goat anti-mouse IgG, Alexafluor 488 goat anti-rabbit IgG, and Alexafluor 594 goat anti-rabbit IgG (Life Technologies) and used at a dilution of 1:500.

Immunofluorescence

At 20 hours postinfection, cells were fixed and permeabilized with -20 ˚C methanol for 5 min and washed with PBS. The cells were then blocked in 10% goat serum for 1 hour. Primary antibody dilutions were added and incubated at room temperature for 1 hour. Cells were washed three times with PBS. Secondary antibodies were added and incubated for 45 min at room temperature in the dark. Cells were washed again in PBS three times. Following staining, nuclei were stained with DAPI (150 μg/mL) for 5 min and washed with PBS. After the third PBS wash, coverslips were mounted to glass slides with Fluoromount-G (SouthernBiotech). Slides were viewed and images were acquired on a Zeiss LSM 710 inverted confocal microscope using ZEISS Efficient Navigation (ZEN) imaging software (black edition).

RESULTS

Pat1b is found in a nuclear pattern in mock-infected and Ad5-infected cells.

Immunofluorescence studies were conducted to visualize whether the cellular protein, Pat1b, was reorganized during an adenovirus infection. As previous studies have shown a disruption of p-bodies at late times during infection (Greer et al. 2011), we infected A549 cells with wild-type Ad5 for 20 hours. We stained for the viral protein, DNA-binding protein (DBP), in order to determine which cells were infected, as well as the cellular proteins, Ddx6 and Pat1b. We also stained for these cellular proteins in mock-infected cells. We found the staining pattern for Ddx6 to be as expected with cytoplasmic foci that became fewer in number after infection and were sometimes found in juxtanuclear aggregates that are assumed to be aggresomes (Figure 1*A*,*C*). We expected to find Pat1b exclusively in cytoplasmic p-bodies; however, in addition to some staining in cytoplasmic foci, we found intense staining in large nuclear aggregates as well as nuclear foci in both mock-infected and infected cells (Figure 1*B*,*D*). The large nuclear aggregates seemed to be localized in regions of the nucleus where the DAPI was absent, suggesting that they might be colocalizing with nucleoli.

Pat1b colocalizes with exogenously expressed Ddx6.

Since the pattern of staining in the mock-infected cells was unexpected, we wanted to verify that the Pat1b cytoplasmic foci were colocalizing with Ddx6. To accomplish this, we transfected A549 cells with a plasmid expressing RFP-tagged Ddx6 and stained for Pat1b. We found that these two proteins did in fact colocalize in distinct cytoplasmic foci (Figure 2*A*). In addition, we infected the transfected cells with wild-type Ad5 to see if overexpression of Ddx6 altered the localization of Pat1b during infection. While we did not have a marker for infected cells in these experiments, other sets of cells infected at the same time with the same virus were found to have >80% of cells expressing DBP and we particularly looked at cells that had aggresome-like foci, which are representative of

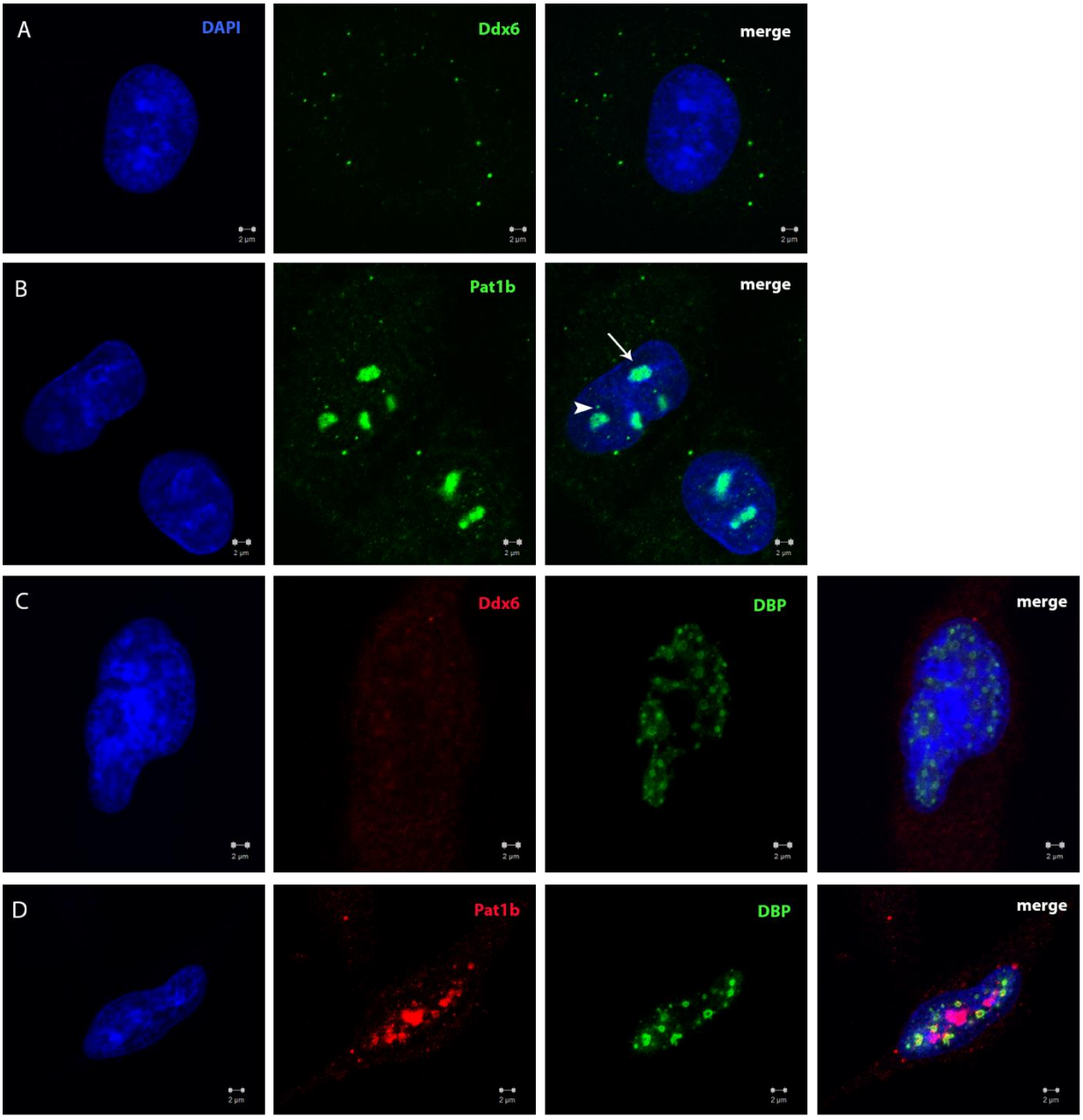

Figure 1. Localization of p-body proteins during an Ad5 infection. A549 cells were either mock-infected (A-B) or infected with wild-type Ad5 virus (C-D) for 20 hours before fixing. Cells were immunostained with antibodies against labeled proteins and stained with DAPI to visualize the nuclei. Merged images are shown in the right panel. Scale bars show 2 μm. The arrow indicates large, nuclear aggregates. The arrowhead indicates nuclear foci.

infected cells. We found Pat1b to again localize in cytoplasmic and nuclear foci as well as in the large nuclear aggregates (Figure 2*B*). Ddx6 and Pat1b were also colocalizing in some cytoplasmic foci. For the most part, Ddx6 cytoplasmic foci colocalized with Pat1b, but there were additional Pat1b cytoplasmic foci that did not colocalize with Ddx6. In addition, when Ddx6 was in larger juxtanuclear aggregates, which are assumed to be aggresomes, Pat1b was also present in these aggregates.

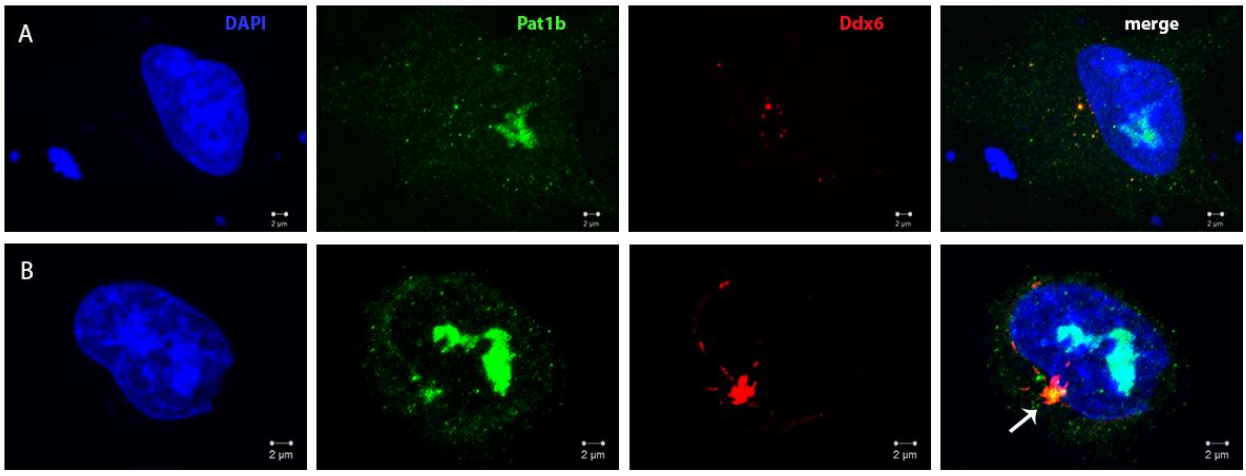

Figure 2. Pat1b localization after exogenous expression of RFP-Ddx6. A549 cells were transfected with a plasmid expressing RFP-tagged Ddx6, incubated for 4 hours, and then either mock-infected (A) or infected with wild-type Ad5 (B) for 20 hours. The cells were then fixed and immunostained with an antibody against Pat1b and stained with DAPI. Merged images are shown in the right panel. Scale bars show 2 µm. The arrow indicates an aggresome.

Pat1b cytoplasmic foci increase in number following Ad infection.

The cytoplasmic foci appeared to be more intense and more numerous in infected cells as compared to mock-infected. In order to quantitate this observation, we counted the number of cytoplasmic foci in 100 cells of either mock-infected cells or cells expressing DBP after 20 hours postinfection. We found that there was an increase in the average number of foci of wild-type Ad5-infected cells with an average of nine foci for infected and 5.6 for uninfected ($p < 0.0001$) (Table I).

Table I. Pat1b cytoplasmic foci increase following E4 11k expression. A549 cells were either mock-infected or infected with wild-type Ad5 or the HA-ORF3 virus for 20 hours. The cells were fixed and immunostained with antibodies against Pat1b and either DBP (Ad5) or HA (HA-ORF3). The cells were also stained with DAPI. For these experiments, the cytoplasmic Pat1b foci were counted in 100 cells (only cells expressing DBP or HA were counted for infected cells). The p value was calculated using a t-test.

	n	**Mean**	**Standard Deviation**	***p* value**
Mock-infected	100	5.6	2.29	
Wild-type Ad5	100	9	4.34	6.39e-11
HA-E4 ORF3	100	11.9	4.4	8.03e-28

E4 11k alone leads to a change in Pat1b localization.

Since E4 11k is necessary and sufficient to disrupt p-bodies as seen by Ddx6, Lsm1, and Ge-1, we wanted to determine if E4 11k was sufficient to increase the number of Pat1b cytoplasmic foci. We infected A549 cells with an E1-replacement virus that only expresses the HA-tagged Ad5 E4 ORF3 gene during infection of a noncomplementing cell line, and does not lead to a productive infection. In these infected cells, the same type of cytoplasmic and nuclear pattern was observed even after transfection with RFP-Ddx6 (Figure 3). When the number of cytoplasmic foci were counted, there was an average of 11.9 ($p < 0.0001$) (Table I). This indicates that E4 11k is sufficient to change the localization pattern of Pat1b by increasing the number of Pat1b cytoplasmic foci.

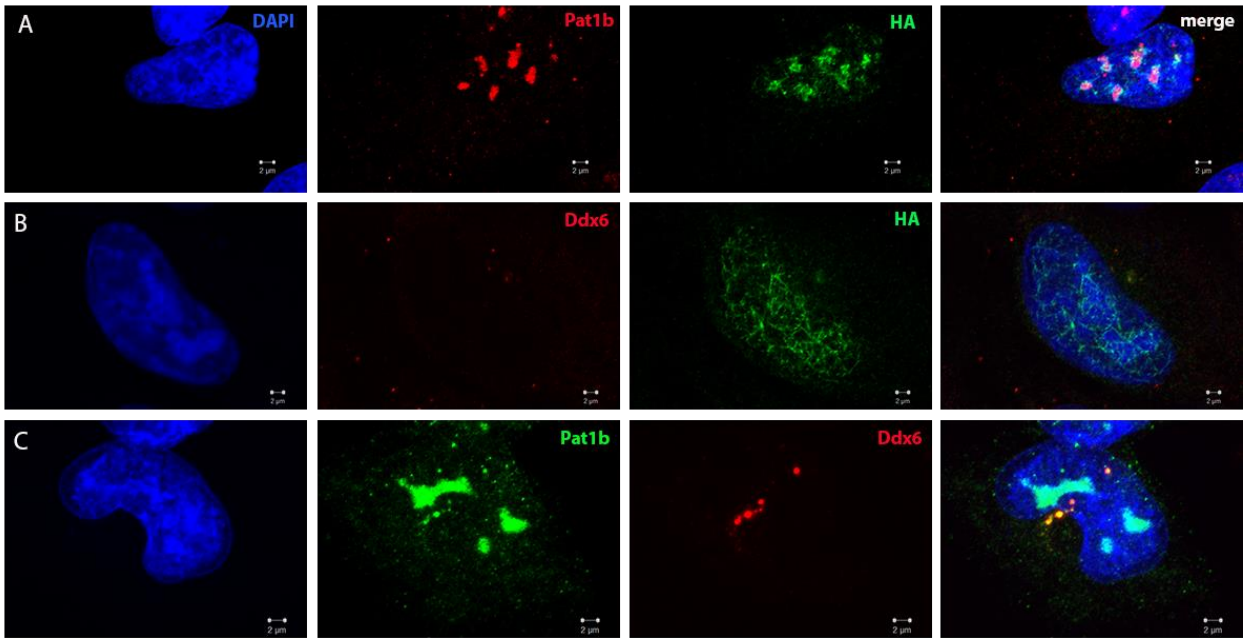

Figure 3. Pat1b localization following E4 11k expression. A549 cells were infected with the E1-replacement HA-E4ORF3 virus for 20 hours and then fixed. The cells were immunostained with antibodies against HA and either Pat1b or Ddx6 (A-B) or Pat1b alone (C). Cells were also stained with DAPI. (C) Prior to infection, cells were transfected with a plasmid expressing RFP-tagged Ddx6 and incubated for 4 hours. Merged images are shown in the right panel. Scale bars show 2 μm.

DISCUSSION

As adenovirus-infected cells have previously been shown to have disrupted p-bodies and Pat1b has also been shown to play a nucleating role in the formation of p-bodies, we wanted to observe the localization of Pat1b during an adenovirus infection. Pat1b interacts with Ddx6 to initiate p-body formation (Ozgur and Stoecklin 2013) and the Ad5 viral protein E4 11k has been shown to have a direct interaction with Ddx6 that leads to the relocalization of Ddx6 to aggresomes (Greer et al. 2011). It is thus possible for Pat1b to remain in cytoplasmic foci even if no other p-body proteins are found in those foci. In fact, this is what we observed during an adenovirus infection. The cytoplasmic foci not only remained in Ad-infected cells, but they became more numerous (Table I). In addition, when cells were transfected with an RFP-tagged Ddx6-expressing plasmid and then infected, there were some Pat1b cytoplasmic foci that colocalized with Ddx6, but also additional Pat1b cytoplasmic foci that did not.

Ddx6 and some other p-body proteins have been shown to decrease in number of cytoplasmic foci when aggresomes are present (Greer et al. 2011). In contrast, we found that Pat1b cytoplasmic foci actually increase in number after infection with wild type Ad5 virus or by expression of E4 ORF3 alone (Table I). The cause for this is not known, but it is possible that the interaction of Pat1b with Ddx6 facilitates the concentration of this protein into fewer, discrete foci. When Ddx6 is disrupted by E4 11k, some Pat1b may travel to aggresomes with Ddx6 while other aggregates of Pat1b may disperse into cytoplasmic foci (Figure 4). However, it has not been ruled out that the increase in cytoplasmic Pat1b foci is due to the stress of infection or protein overexpression.

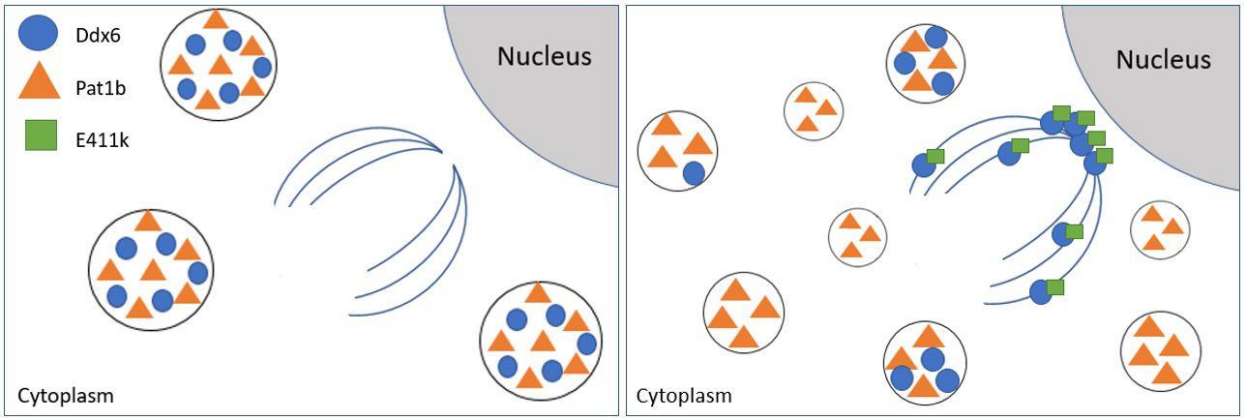

Figure 4. Cytoplasmic foci are disrupted by E4 11k during adenovirus infection. E4 11k-dependent disruption of p-bodies results in a greater number of smaller Pat1b foci. Ddx6 is bound by E4 11k and travels along microtubules to form aggresomes. Pat1b is found in all Ddx6 foci, but not all Pat1b foci colocalize with Ddx6. Some Pat1b is found in aggresomes with Ddx6.

Based on the phenotype of single- and double-mutant viruses of the E4 ORF3 and E1B 55k genes, these proteins play a role in stimulating late viral gene expression and inhibiting cellular protein synthesis (Shepard and Ornelles 2004). The disruption of the mRNA regulatory p-bodies by E4 11k may play a role in the control of late gene expression in infected cells. The mechanism of this control and the key cellular proteins involved is not well understood and will be the subject of further research. It is unclear if Ddx6 or Pat1b play a specific role or if it is a generalized disruption of p-bodies that is important. Potentially, Pat1b or Ddx6 play a role in selective decapping of viral mRNAs and the disruption of p-bodies is important to increase late viral gene expression. Alternatively, the disruption of p-bodies may allow the virus to co-opt the decapping proteins for their own purposes and cause decapping of cellular mRNAs. Ddx6 and Pat1b have also been shown to play important roles in translation and replication during a hepatitis C virus infection (Scheller et al. 2009). These proteins, along with another p-body protein, Lsm1, bind to the 5' and 3' untranslated regions (UTRs) of the viral genome, leading to efficient translation.

The other interesting finding in our studies is the nuclear localization of Pat1b in A549 cells. It is unclear exactly where in the nucleus the large aggregates of Pat1b are localizing. They appear to localize to regions where the DAPI staining is absent, suggesting that they are colocalizing with the nucleoli. There is some previous evidence of Pat1b in nucleolar caps, but only after treatment with leptomycin B to inhibit nuclear export (or with an NES-mutated Pat1b) and actinomycin D to inhibit transcription (Marnef et al. 2012). When only nuclear export was inhibited, Pat1b was found in nuclear speckles and PML nuclear bodies. We intend to further study the nuclear staining that we observed in mock-infected and infected cells. Possibly, this is a phenomenon of this particular cell line, A549s, potentially indicating a mutation in the NES of Pat1b in these cells. Another explanation could be that there is another nuclear protein that cross-reacts with the Pat1b antibody. We intend to perform a Western blot to confirm the target of this Pat1b antibody and visualize any other cross-reactive bands that may appear. We will also attempt to express a tagged version of Pat1b exogenously to confirm the current results.

Overall, we have shown an interesting localization pattern of Pat1b in both mock-infected and adenovirus-infected cells. The nuclear staining pattern needs further study to confirm. However, the change in number of Pat1b cytoplasmic foci following wild-type Ad5 infection or E4 11k expression alone indicates an additional p-body protein disruption pattern. Other p-body proteins, such as Ddx6 and Lsm1, have been found to decrease in number following infection and aggresome formation, whereas Ge-1 cytoplasmic foci remained the same. However, Pat1b cytoplasmic foci appear to increase in number. The purpose for this is not clear, but it adds an interesting layer to the role of p-body disruption in control of gene expression during a late Ad5 infection.

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