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THE ROLE OF ADENOVIRUS SEROTYPE 5 E4 11K IN THE RELOCALIZATION OF CELLULAR P BODY PROTEINS

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ABSTRACT

Human adenoviruses are a useful tool to understand basic cellular biology in addition to viral infections. Historically, cellular splicing was first discovered in adenovirus, but other cellular processes, such as double-strand break repair and aggresome formation, have been further elucidated through adenoviral infection. The adenovirus protein E4 11k has been shown to disrupt cytoplasmic processing bodies (p bodies), which are not well-understood but are involved in mRNA metabolism. Several p body proteins were found to be reorganized in the cytoplasm with adenovirus serotype 5 (Ad5) able to cause the colocalization of these p body proteins with aggresomes. The p body protein Lsm1 has been found to colocalize with E4 11k in aggresomes, but Edc3 and Pat1b are two p body proteins that have not been as well-studied in adenovirus-infected cells. Cells were treated with cadmium chloride or infected with wild-type and mutant viruses before staining them to visualize the p body proteins and a marker for viral infection by confocal microscopy. We were able to determine that the presence of E4 11k was not necessary for relocalization of either Lsm1 or Edc3 to aggresomes, while Pat1b did not localize to aggresomes under any conditions tested. We wanted to characterize Pat1b relocalization further so we counted the number of Pat1b foci in mock-infected cells and then the virally-infected cells, and were able to determine that Ad5 E4 11k is necessary and sufficient to induce an increase in cytoplasmic Pat1b foci. We also determined that this increase in Pat1b foci appears to be serotype-specific when compared to Ad9 and Ad12 E4 11k protein. The lack of apparent change with Pat1b localization with Ad9 E4 11k expression was a novel finding that suggests there may be something unique about Pat1b and p body localization.

Keywords: adenovirus, Pat1b, Edc3, Lsm1, p bodies, serotypes, viral infection

INTRODUCTION

Human adenoviruses can cause a range of diseases in humans depending on the serotype. These include respiratory infections ranging from the common cold to the rare development of acute respiratory distress syndrome (ARDS), conjunctivitis, or gastroenteritis. There are over 100 different human adenovirus genotypes discovered so far (n.d.). Respiratory illnesses are the major type of disease that adenoviruses cause resulting in 2-5% of respiratory illnesses among humans. More serious disease can occur in immunocompromised patients, particularly in transplant patients (Lion 2014). Due to the diversity of genotypes and the primarily self-limiting infections that are caused, the focus of adenovirus research is more on using adenovirus as a model for other viral infections and for studying cellular biology.

All viruses must disrupt some cellular processes in order to replicate. Most RNA viruses replicate in the cytoplasm and thus primarily disrupt cytoplasmic processes. Most

DNA viruses replicate their genomes in the nucleus but need to disrupt cellular processes that take place in both the nucleus and cytoplasm. Adenovirus has a double-stranded (ds) DNA genome and replicates its genome in the nucleus. It has viral proteins that will drive the cell into the S phase of the cell cycle so that cellular DNA replication machinery is present in the nucleus (Ben-Israel and Kleinberger 2002). It also disrupts the double-strand break (DSB) repair pathway to prevent the cell from recognizing the linear dsDNA genome as a DSB and ligating it together with deletions at the ends of the genome (Kleinberger 2020). For example, the adenovirus early protein, E4 11k, rearranges PML nuclear bodies, which are discrete nuclear structures that contain a variety of proteins, into elongated track-like structures. In doing so, it sequesters cellular proteins, such as Mre11-Rad50-Nbs1, which forms a complex (the MRN complex) that is a sensor of DSBs, presumably to prevent these proteins from binding to the linear ends of viral dsDNA as it is replicated (Evans and Hearing 2003).

Viral gene expression is necessarily controlled in a temporal manner. Some viruses, like adenovirus, have immediate early proteins that are expressed first and are involved in activating transcription of early viral genes. These early proteins are denoted with an E and then a number based on which region of the genome it is located. E1A is the immediate early protein in adenovirus and it activates some cellular genes, such as those involved in S phase, in addition to the early viral genes (Berk 2013). The early genes are those that disrupt cellular processes to make the cellular environment more amenable to viral growth and prepare the cell for viral DNA replication. In the late phase of viral replication, structural genes that make up the virion structure and genes involved in maturation and assembly are expressed. The cellular environment is altered by this point to primarily express viral proteins while shutting down host protein synthesis.

It is known that multiple adenoviral proteins are involved in shutting down host protein synthesis while stimulating late viral protein synthesis, including E4 11k and E1b 55k (Shepard and Ornelles 2004). E1b 55k works together with E4 34k, another viral early protein, as an E3 ubiquitin ligase complex that functions to target specific cellular proteins for degradation (Harada et al. 2002). The mechanisms of control of late gene expression for these proteins are unknown but the discovery that E4 11k disrupts cytoplasmic processing bodies (p bodies) (Greer et al. 2011) may provide a link. P bodies are cytoplasmic aggregates of cellular RNA and proteins that are assumed to play a role in RNA degradation and miRNA-mediated translational inhibition based on their composition. There are exonucleases and decapping enzymes that are involved in RNA degradation as well as proteins involved in binding to and processing miRNAs made by the cell in order to target specific mRNAs for inhibition of translation. More research is necessary to determine the function of the E4 11k-dependent disruption of p bodies late during infection.

E4 11k has been shown to disrupt several p body proteins, including Ddx6, Lsm1, Ge-1, Xrn-1, and Ago2 (Greer et al. 2011). Additionally, E4 11k from adenovirus serotype 5 was shown to relocalize these p body proteins to aggresomes, which are cytoplasmic structures that form under periods of cellular stress at the microtubule organizing center and are marked by the presence of gamma-tubulin. E4 11k proteins from other serotypes were able to disrupt p body proteins, but they did not relocalize them to aggresomes. The chemical stressor, cadmium chloride, however, has been shown to relocalize both Lsm1 and Ge-1 to aggresomes. Under the same conditions, Ddx6, was not shown to be relocalized by cadmium chloride. Interestingly, when Ddx6 was found in aggresomes

during viral infection, the number of p bodies containing Ddx6 decreased in number (Greer et al. 2011). Previously Ddx6 has been shown to physically interact with another p body protein, Edc3, which acts by decapping mRNAs (Coller and Parker 2005). Pat1b has been found to be an important scaffolding protein, along with Ddx6, in the formation of p bodies (Ozgur and Stoecklin 2013). More recently, Pat1b has been visualized during an adenovirus infection and the cytoplasmic Pat1b foci increased in cells infected with wild type adenovirus serotype 5 (Ad5) and a virus that only expresses an HA-tagged Ad5 E4 11k protein (Friedman and Karen 2017). This HA-tagged protein has been fused with a short chain of amino acids from the hemagglutinin protein of influenza so it can be easily detected with an anti-HA antibody.

We decided to further explore the disruption of certain p body proteins during an adenovirus infection. We compared the localization of p body proteins during an infection with cadmium chloride treatment as only a few p bodies proteins had been visualized under such conditions with varying results (Greer et al. 2011). This allowed us to determine whether additional p body proteins relocalized to aggresomes in response to cadmium chloride, like Lsm1 and Ge-1, or remained as p bodies, like Ddx6. We also infected with an additional virus that is deleted in the gene that encodes E4 11k (E4 ORF3) to determine that the E4 11k protein is necessary as well as sufficient to induce the change in Pat1b localization. Additionally, we used HA-tagged E4 11k-expressing viruses from two other serotypes from divergent groups to determine the serotype-specificity of the Pat1b disruption at late times during an infection. Our research finds that the presence of Ad5 E4 11k was not necessary for the relocalization of Lsm1 or Edc3 to aggresomes and Pat1b did not relocalize to aggresomes under either condition. Additionally, Ad5 E4 11k is necessary and sufficient to increase the number of cytoplasmic Pat1b foci in a serotype-specific manner.

MATERIALS AND METHODS

Cell Culture: A549 and HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with the addition of 10% calf serum, glutamine, penicillin, and streptomycin. The cells were incubated in 5% CO_2 at 37° C, and they were passaged at 80-90% confluency. One day before infection, the cells were split onto coverslips in a 24-well plate.

Viruses, Infection, and Treatments: Viruses used for these infections include wild-type adenovirus serotype 5 (Ad5), Ad5 E4 11k deletion mutant (*dl*1013) (Bridge and Ketner 1989), and E1-replacement viruses where the E1 gene was replaced with an HA-tagged E4 ORF3 (early region 4 open reading frame 3) gene under the control of a CMV (cytomegalovirus) promoter (during infection of A549 or HeLa cells, only the E4 ORF3 gene is expressed, resulting in the production of the E4 11k protein). There were three E1 replacement viruses with the Ad5, Ad9, or Ad12 HA-tagged E4 ORF3 gene. These viruses were grown in either HeLa cells (wild type and *dl*1013) or HEK-293 cells (complementing cell line for the E1-replacement viruses) and purified either by cesium chloride gradient or an Adenovirus Purification Kit (Biomiga). Cells were infected at a ratio of about 200 virus particles per cell and infected for 30 hours.

For cadmium chloride treatment, a 20mM stock solution was made using distilled water. Each well had a final concentration of 30 μM cadmium chloride supplemented for 6 hours before being fixed and stained for immunofluorescence.

Antibodies: The primary antibodies that were utilized in this experiment were an anti-HA mouse monoclonal (clone 16B12) at 1:200, an anti-Pat1b rabbit polyclonal (Abnova) at 1:300, an anti-Edc3 rabbit polyclonal (Invitrogen) at 1:500, an anti-Lsm1 mouse monoclonal (Invitrogen clone OTI9D7) at 1:500, an anti-gamma tubulin mouse monoclonal (Invitrogen clone 4D11) at 1:2000, an anti-gamma tubulin rabbit polyclonal (Novus) at 1:2000, and an anti-DBP mouse monoclonal (B6-8) at 1:100. The secondary antibodies used were Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen).

Cell Fixation and Immunofluorescence: At 30 hours post-infection or 6 hours post treatment, the cells were fixed and stained. The cells were washed in phosphate-buffered saline (PBS) three times, fixed with ice-cold methanol for 5 minutes, and then were washed again in PBS three times. The cells were then blocked in 10% goat serum for 1 hour. Primary antibodies were added and incubated at room temperature for 1 hour. The cells were washed again with PBS three times and then the secondary antibodies were added and incubated at room temperature for 45 minutes in the dark. Finally, the cells were washed with PBS three more times, followed by the application of DAPI to stain cell nuclei, and three more PBS washes. These cells were then mounted on coverslips using Fluoromount G and observed utilizing an Olympus FV3000 confocal microscope.

Analysis of Pat1b foci: For each condition in the Pat1b-foci experiments, the Pat1b foci from 100 cells were counted from two independent experiments with the observer scoring foci blindly. These data were subjected to ANOVA analysis to determine if there were significant differences between the groups. Additionally, the Tukey's post-hoc test was performed to determine which groups were significantly different.

RESULTS

Ability of cadmium chloride treatment to relocalize Lsm1, Edc3, and Pat1b to aggresomes

To determine if chemical stress is sufficient to relocalize Lsm1, Edc3, and Pat1b to aggresomes, the cells were either mock-infected, infected with wild type Ad5, or treated with $CdCl_2$. The relocalization of the p bodies to aggresomes only during a wild type infection would suggest that E4 11k may possess a specific mechanism for the relocalization of those proteins. If the p body protein is also relocalized during cadmium chloride treatment, then E4 11k may not be necessary for the relocalization to aggresomes as the stress of an infection may be able to induce the localization. Lsm1 and Edc3 were relocalized to aggresomes, as seen by colocalization with gamma-tubulin in large, juxtanuclear foci, in both wild type adenovirus infection and under $CdCl_2$ treatment (Figures 1 and 2). Pat1b was not found to be relocalized to aggresomes under either condition (Figure 3).

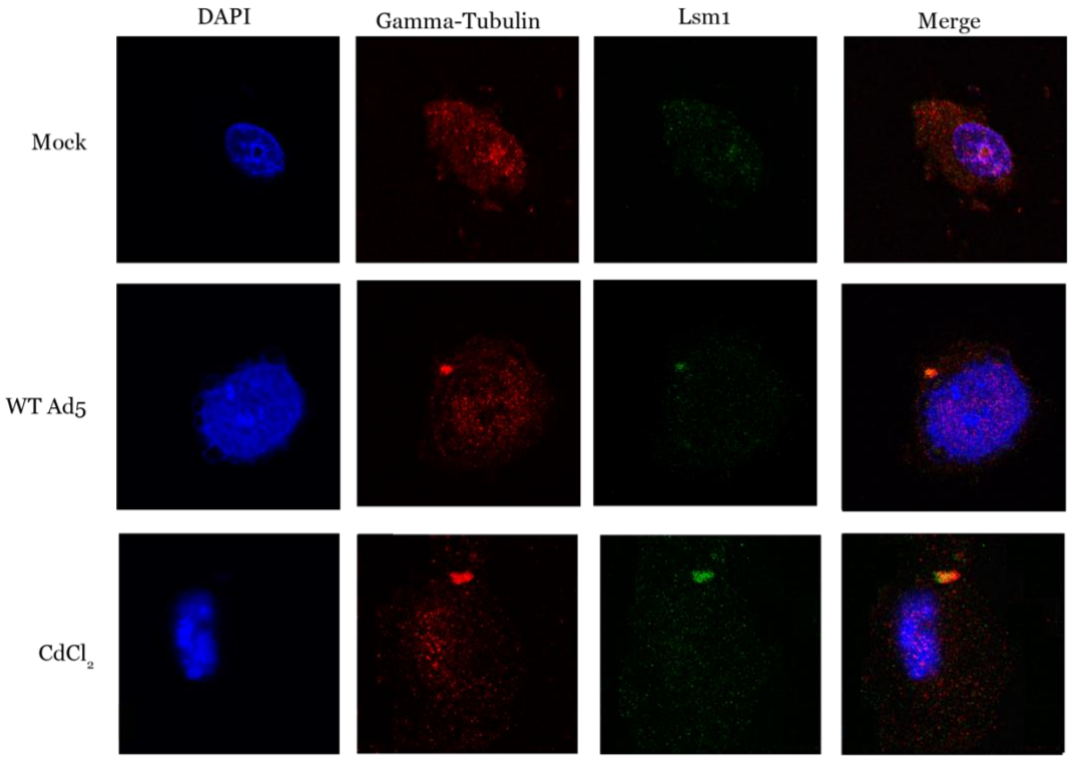

Figure 1. Lsm1 is found in aggresomes during an adenovirus infection and during chemical stress. HeLa cells were mock-infected, infected with wild type Ad 5 virus, and treated with 30 μM CdCl2. The cells were immunostained to visualize γ-tubulin and Lsm1 and then DAPI stained to visualize cellular DNA. Images are representative of three trials.

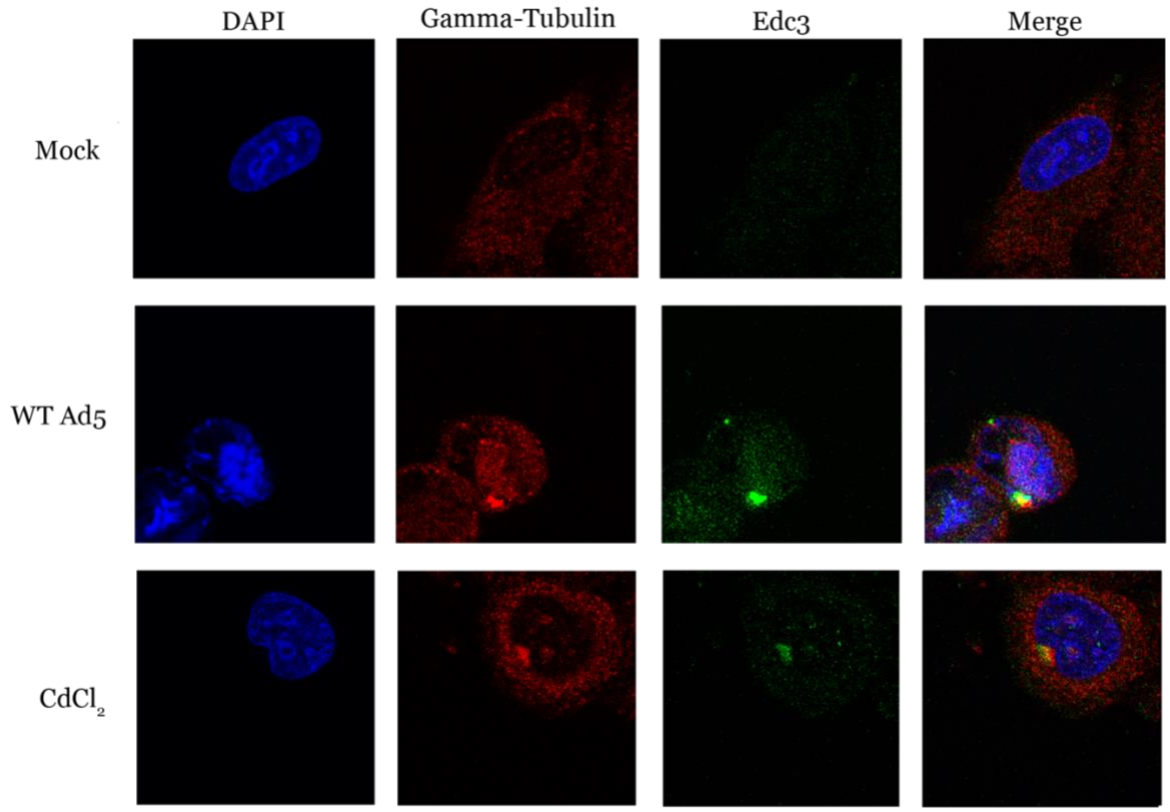

Figure 2. Edc3 is found in aggresomes during an adenovirus infection and during chemical stress. HeLa cells were mock-infected, infected with wild type Ad 5 virus, and treated with 30 μM CdCl2. The cells were immunostained to visualize γ-tubulin and Edc3 and then DAPI stained to visualize cellular DNA. Images are representative of three trials.

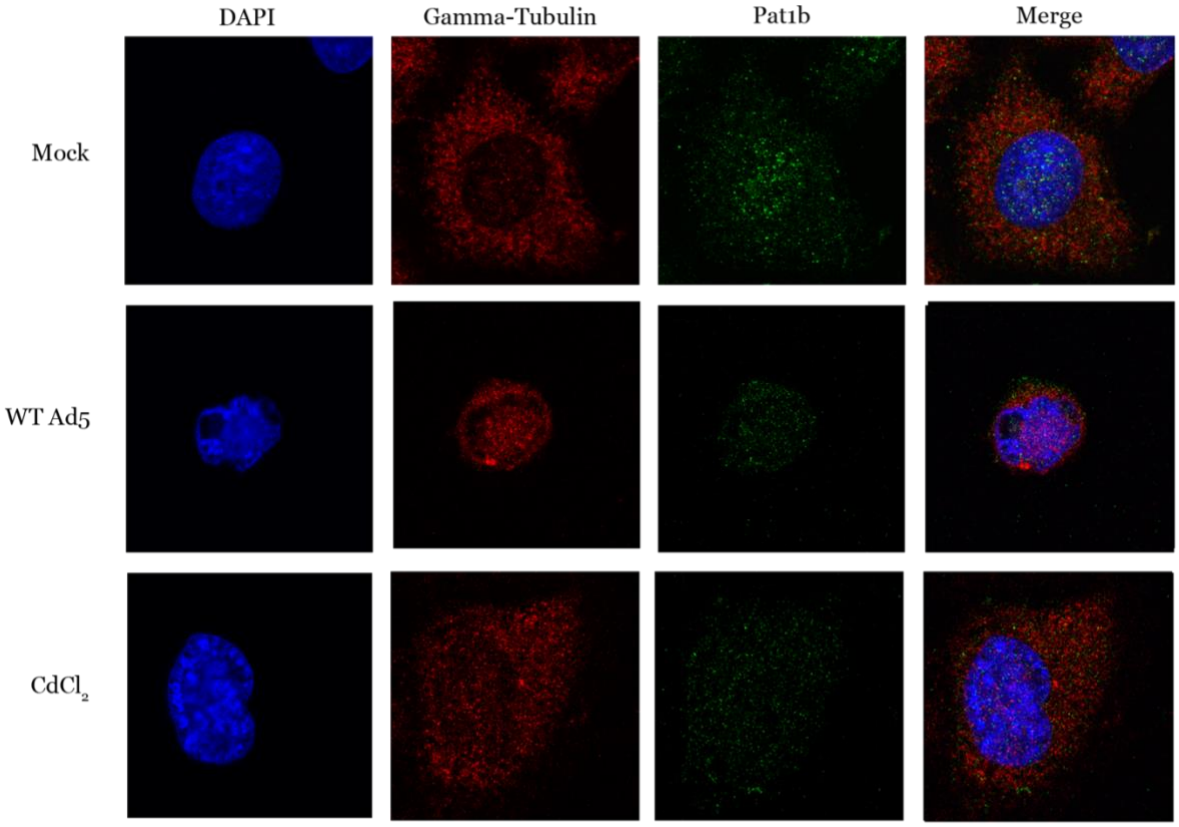

Figure 3. Pat1b is not found in aggresomes either during an adenovirus infection or during chemical stress. HeLa cells were mock-infected, infected with wild type Ad 5 virus, and treated with 30 μM CdCl2. The cells were immunostained to visualize γ-tubulin and Pat1b and then DAPI stained to visualize cellular DNA. Images are representative of three trials.

Adenovirus E4 11k is necessary and sufficient to increase the number of Pat1b foci

Previous studies had shown an increase in cytoplasmic Pat1b with wild-type adenovirus serotype 5 and an E1-replacement virus expressing only Ad5 HA-tagged E4 11k protein (Friedman and Karen 2017). These results showed that Pat1b foci increase in number under wild-type conditions and that E4 11k is sufficient to direct this increase. We wanted to replicate these findings and include an additional virus where E4 11k was deleted in order to determine if E4 11k was also necessary for the increase in Pat1b foci. There are several redundant functions between E4 11k and the E1b 55k and E4 34k proteins, so the deletion mutant virus would be able to answer whether there are other mechanisms within wild-type virus that could lead to the change in Pat1b localization. As the E4 11k protein is encoded by E4 ORF3 gene, if E4 11k is necessary to increase Pat1b foci, then infection with the *dl*1013 virus (deletion of the E4 ORF 1-3 genes, see Figure 4) would result in numbers of foci similar to mock-infected cells that are not infected with any virus.

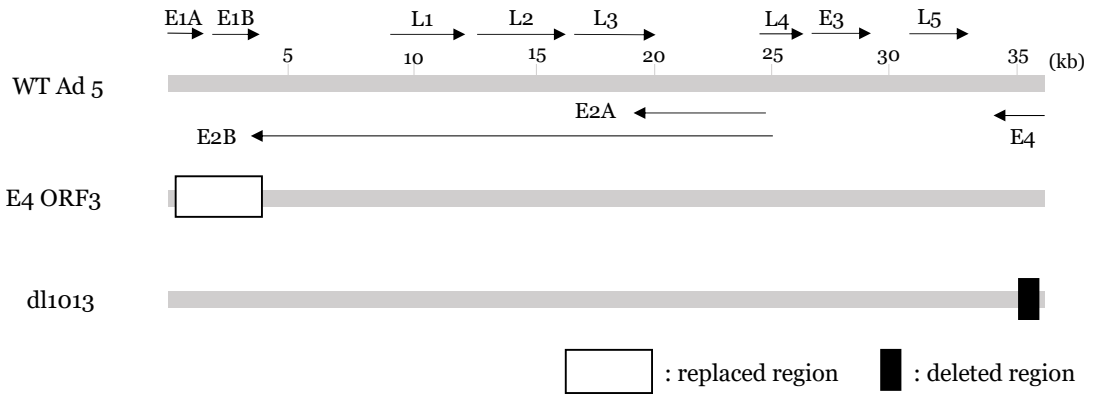

Figure 4. Schematic representation of the genomes of the adenoviruses used in this study. The wild type adenovirus is shown as a reference. The E4 ORF3 virus had the E1 region replaced with an HA-tagged E4 ORF3 gene under the control of the CMV promoter. The mutant virus, *dl*1013, has a deletion of the E4 ORFs1-3. The open box indicates the replaced region while the filled in box indicates the deletion.

As seen previously, mock-infected cells had an average of 4.6 cytoplasmic Pat1b foci per cell while wild-type Ad5-infected cells had an increase in Pat1b foci with an average of 7.5 foci per cell after 30 hours of infection (Figures 5 and 6). Additionally, E4 11k was shown to be sufficient for an increase in cytoplasmic Pat1b foci with 9.1 foci per cell infected with the E4 11k-expressing virus. Finally, with the E4 11k-deleted virus (*dl*1013), the number of cytoplasmic Pat1b foci remained similar to mock-infected cells with an average of 4.4 foci per cell. An ANOVA was performed with the Tukey's post-hoc test to show that there was a significant difference between the number of Pat1b foci in mock-infected cells and wild-type and E4 11k-infected cells, but not between mock and the E4 11k-deleted virus infected cells (Figure 6). Additionally, the E4 11k-expressing virus had significantly more Pa1b foci than the wild-type virus. The E4 ORF3 gene is under the control of a CMV (cytomegalovirus) promoter, which may result in higher levels of E4 11k protein than in the wild-type infection. This may be the reason for the further increase in the number of Pat1b foci in these cells.

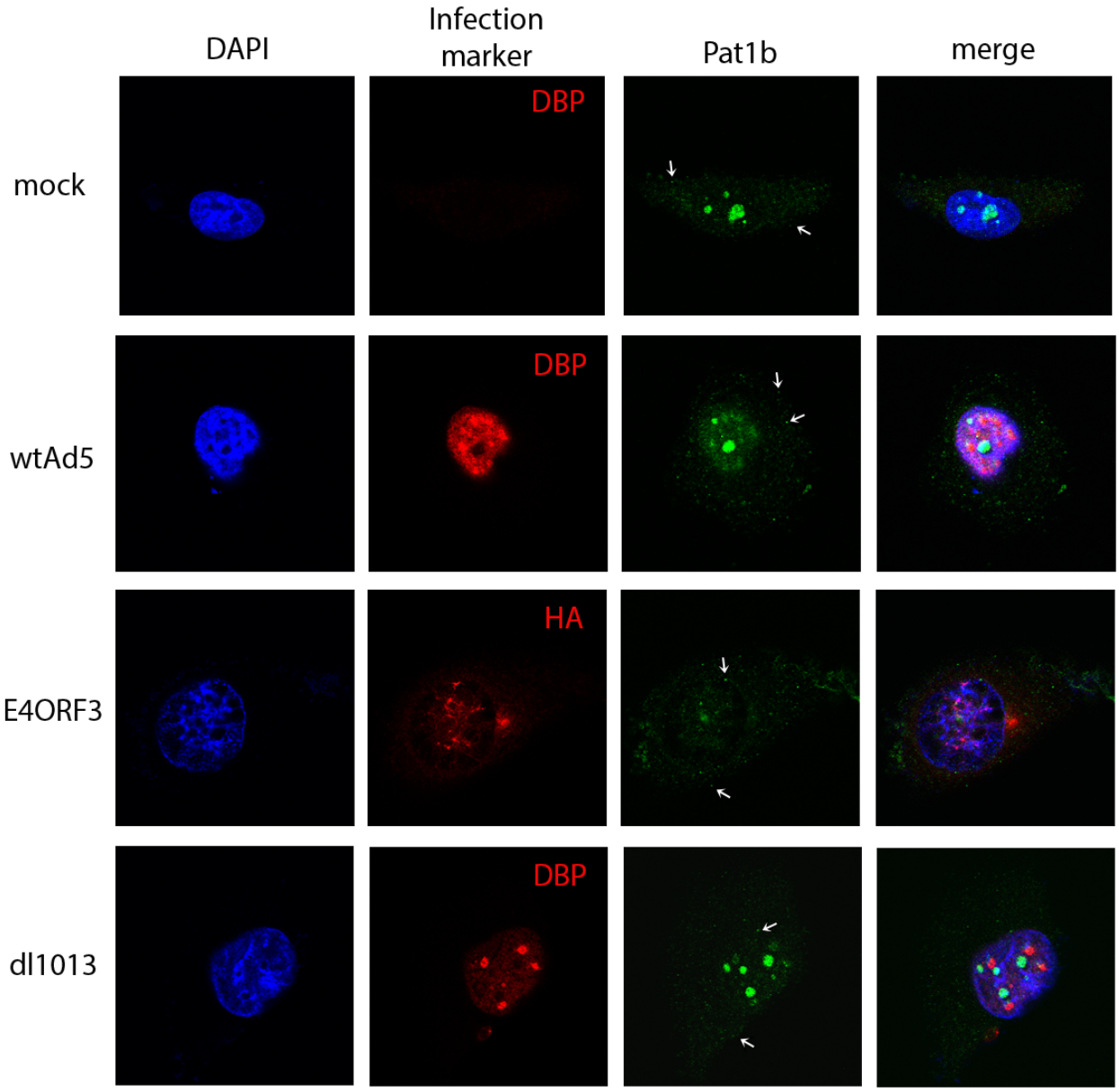

Figure 5. Immunofluorescence of Pat1b during infection with adenovirus. A549 cells were mock-infected (A), infected with wild-type Ad5 virus (B), infected with the E1-replacement virus expressing only the E4 ORF3 gene (C), or the E4 ORF3-deleted virus (*dl*1013) (D). Cells were fixed at 30 hours post-infection (hpi) and immunostained for DBP (B and D) or HA (C), and Pat1b. Nuclei were stained with DAPI. Arrows indicate Pat1b foci that were counted.

While Pat1b is primarily observed in the cytoplasm, nuclear aggregates were observed by immunostaining for Pat1b. When cells were infected with wild-type adenovirus and the mutant, *dl*1013, there appeared to be colocalization of Pat1b with DBP. Additional studies were performed without staining for DBP (data not shown), and that pattern of staining was not observed, suggesting it is derived from fluorescence that has

bled through from one channel to the other. Under other conditions, large nuclear aggregates were observed in mock and infected cells, suggesting that it is not a result of adenovirus infection or expression of E4 11k. A YFP-Pat1b construct was obtained (Ozgur and Stoecklin 2013) and cells were transfected with this plasmid and stained for Pat1b. While there was no nuclear localization of the YFP-Pat1b, the Pat1b immunostaining colocalized with the cytoplasmic YFP-Pat1b (data not shown). Taken together, these data suggest that the nuclear staining seen with Pat1b is an artifact or a result of non-specific staining by the antibody, while the cytoplasmic foci do represent p bodies.

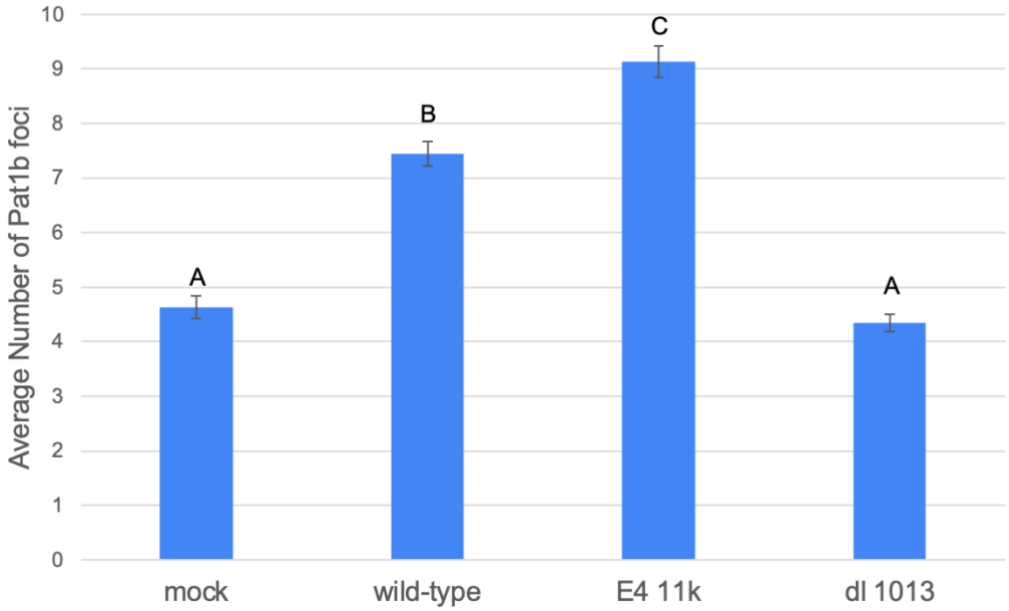

Figure 6. Quantification of Pat1b during infection with adenovirus. A549 cells were infected and stained as in Figure 2. The number of Pat1b foci were counted in 30 infected cells under each condition. This was performed in duplicate and combined averages were plotted with error bars representing standard error. Differences were determined by one-way ANOVA and Tukey's test. The different letters above each bar indicate significant differences; any bars with the same letter are not significantly different from each other while bars with different letters have a p value less than 0.5. For example, mock and dl1013 have the same letter (A) and thus are not significantly different while mock and wild type have different letters (A and B) and are significantly different from each other.

Serotype specificity of the E4 11k-dependent relocalization of Pat1b

As the Ad5 E4 11k protein was shown to be necessary and sufficient for the increase in Pat1b foci, we wanted to explore this relocalization further. Previous studies had indicated that some of the Ddx6 relocalization characteristics had been serotype-specific (Greer et al. 2011). While all adenovirus serotypes tested showed disruption of p bodies, it was only Ad5 E4 11k that relocalized p body proteins to aggresomes and was shown to bind to it directly via a co-immunoprecipitation assay. We decided to infect with E1-replacement viruses that expressed the HA-tagged E4 11k protein from Ad9 and Ad12 in addition to Ad5. These represent 3 different subgroups of adenovirus that are more distantly related to each other.

For this experiment, mock-infected cells and Ad5 E4 11k-expressing virus infected cells were repeated with the average number of cytoplasmic Pat1b foci being 3.6 and 8.1, respectively (Figures 7 and 8). When we infected with the Ad9 E4 11k- and Ad12 E4 11k-expressing viruses, we found that the average number of Pat1b foci were 4.3 and 4.6, respectively. The ANOVA and Tukey's test showed that there was a significant difference between mock and Ad5 E4 11k, as seen previously (Figure 8). The number of foci for the other serotypes, Ad9 and Ad12, were much closer to mock, however, the Ad12 E4 11k showed significantly higher numbers compared to mock ($p = 0.0029$). There was no difference between Ad9 and mock or Ad12, but there was a significant difference between Ad5 and Ad9 or Ad12.

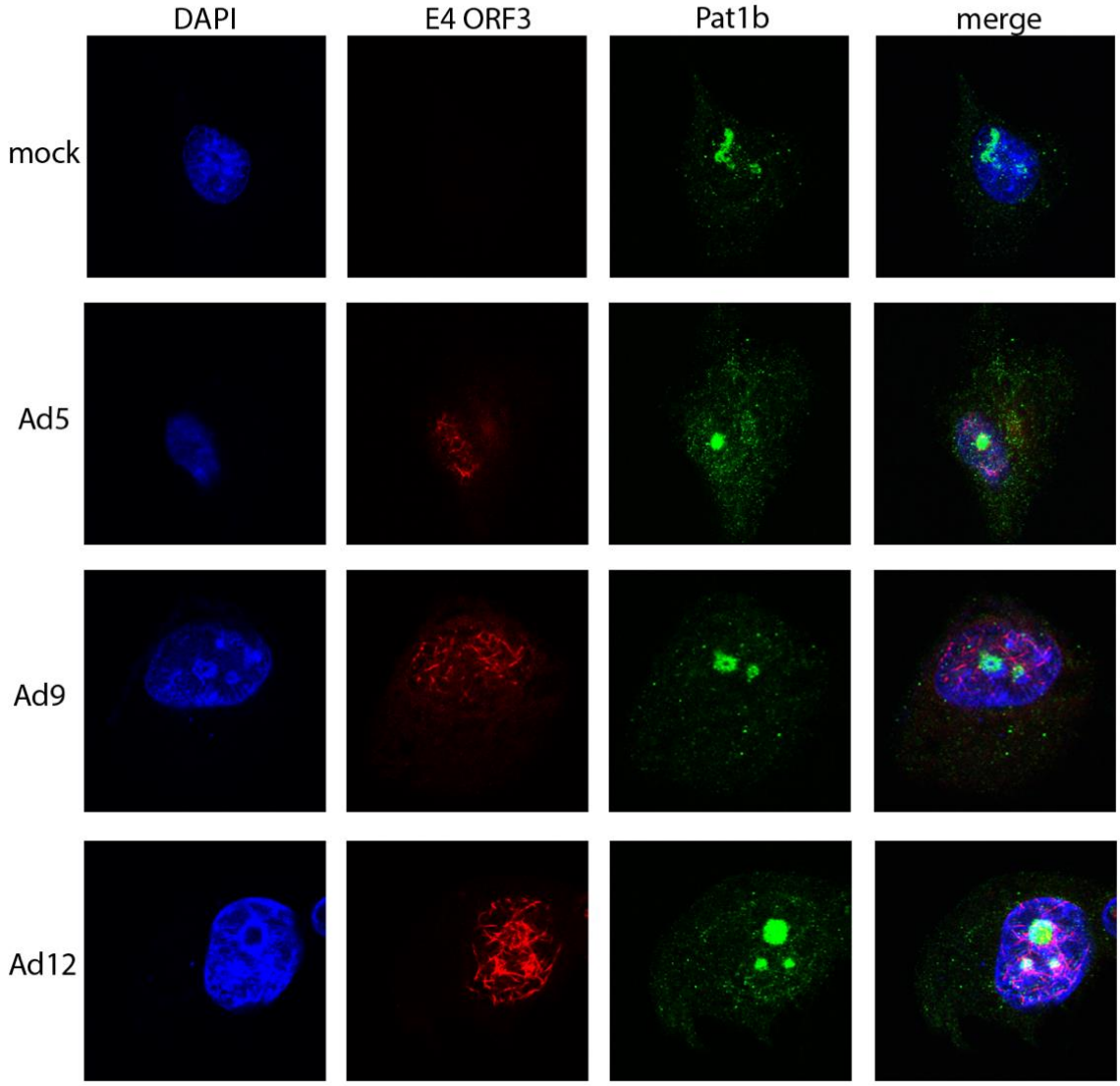

Figure 7. Immunofluorescence of Pat1b during infection with different serotypes of adenovirus E4 11k. A549 cells were mock infected (A), infected with Ad5 E4 ORF3 (B), infected with Ad9 E4 ORF3 (C) or infected with Ad12 E4 ORF3 (D). Cells were fixed at 30 hours post infection (hpi) and immunostained for HA and Pat1b. Nuclei were stained with DAPI.

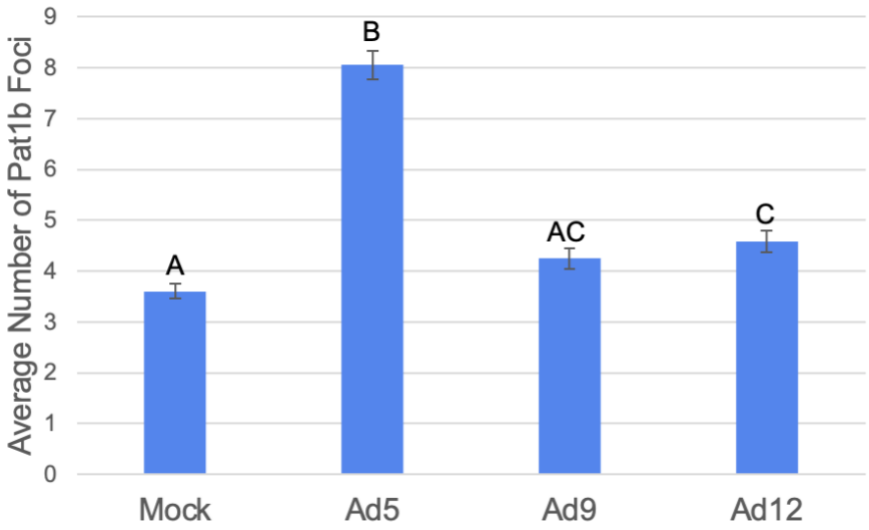

Figure 8. Quantification of Pat1b during infection with different serotypes of adenovirus E4 11k. A549 cells were infected and stained as shown in Figure 3. Pat1b foci were counted in 30 mock-infected and cells infected with E411k-expressing virus. The experiment was performed in duplicate and the combined averages were graphed with error bars representing standard error. A one-way ANOVA and Tukey`s test was used to indicate any significant differences between any two groups. The different letters above each bar indicate significant differences; any bars with the same letter are not significantly different from each other while bars with different letters have a p value less than 0.5. For example, as Ad9 shares letters (A and C) with both mock and Ad12, it is not significantly different from either of them, while it does not share a letter with Ad 5 (B) so it is significantly different from that virus.

DISCUSSION

There are many functions attributed to the E4 11k protein of adenovirus; some of which are conserved across serotypes and other functions are serotype-specific. The conserved functions of E4 11k include reorganizing PML nuclear bodies, sequestering the sensor of double-strand breaks, the MRN complex, in nuclear tracks, inhibiting the interferon response via PML disruption, and binding to and redistributing TIF1 alpha (Carvalho et al. 1995; Evans and Hearing 2003; Hösel et al. 2001; Ullman et al. 2007; Yondola and Hearing 2007). Nonconserved functions include aggresome formation and binding to Ddx6 and redistributing it to aggresomes (Blanchette et al. 2013; Greer et al. 2011)

Some p body proteins had been shown to be relocalized to aggresomes during wild type adenovirus 5 infection, but we sought to determine if E4 11k was necessary for this relocalization in three specific p body proteins; Lsm1, Edc3, and Pat1b. In the case of Lsm1 and Edc3, both p body proteins were shown to relocalize to aggresomes under both chemical stress (caused by cadmium chloride) and Ad 5 infection indicating that E4 11k is not necessary for their relocalization (Greer et al. 2011). In the case of Pat1b, however, the protein was not shown to relocalize to aggresomes under either condition. Lsm1 had previously been shown to relocalize to aggresomes following cadmium treatment (Greer et al. 2011) so our results confirm these earlier studies. Edc3 had not been previously observed following cadmium treatment, but as it followed the same pattern as most other p body proteins observed (Lsm1 and Ge-1), it suggests that movement of p body proteins to aggresomes following chemical stress is common.

The only other p body protein that had been observed following cadmium treatment was Ddx6 and while it did colocalize with aggresomes during an adenovirus

infection, it was not found in aggresomes following cadmium treatment (Greer et al. 2011). The conclusion was that the movement of Ddx6 to aggresomes was E4 11k-dependent and the fact that Ddx6 and E4 11k were found to interact via a co-immunoprecipitation assay strengthened that conclusion (Greer et al. 2011). Interestingly, Pat1b did not follow either pattern. While it did not colocalize with aggresomes following cadmium treatment similar to Ddx6, it also did not colocalize with aggresomes during an adenovirus infection. Previous experiments had shown Pat1b colocalizing in large, juxtanuclear aggregates with Ddx6, however, the structures had not been confirmed as aggresomes by colocalization with gamma-tubulin, and they were only seen with an overexpression of RFP-tagged Ddx6 (Friedman and Karen 2017). It is possible that a small amount of Pat1b does localize to aggresomes during infection and/or chemical stress but it is too small an amount to visualize. On the other hand, it may also be that an overexpression of Ddx6 is able to artificially pull Pat1b with it to aggresomes and that this would not be seen under normal conditions where Ddx6 is only expressed endogenously.

As E4 11k had been shown to relocalize many p body proteins, but the location of Pat1b during an adenovirus infection had not been well-examined, we aimed to determine the localization of Pat1b given the recent discovery of its scaffolding role in the nucleation of p bodies. Initial studies had been done to show an increase in cytoplasmic Pat1b foci with some of those foci colocalizing with Ddx6 (Friedman and Karen 2017). Studies had shown that E4 11k expression was sufficient to lead to this increase, but given the redundancy in some functions of E4 11k with other viral proteins, we went further to determine that E4 11k is also necessary to increase the number of Pat1b foci. These data suggest that the relocalization of Pat1b during late times in an adenovirus infection is E4 11k-dependent.

Additionally, we went on to show that this relocalization of Pat1b is primarily serotype-specific. The E4 11k protein from Ad9 did not show a significant increase in Pat1b foci, indicating that it is not necessary for Ad9 to replicate. It is possible that the function of this relocalization is redundant with other adenoviral proteins alleviating the reliance on Pat1b disruption by Ad9. We did see a slight increase in the number of Pat1b foci when the E4 11k protein of Ad12 was expressed, indicating that the changes to p body structure may be more subtle in some serotypes than others, but that there are still changes being observed. Greer *et al.* (2011) showed that the E4 11k protein from several different serotypes of adenovirus (Ad3, Ad4, Ad5, Ad9, and Ad12) was able to disrupt several p body proteins. Only Ad5 E4 11k was able to relocalize these p body proteins to aggresomes and bind directly to one p body protein in particular, Ddx6. The lack of noticeable relocalization of Pat1b with Ad9 E4 11k is thus a novel observation.

A model of E4 11k-dependent disruption of p bodies had been proposed to explain the observations made following the discovery of the localization pattern of Pat1b (Friedman and Karen 2017). It was proposed that Ad5 E4 11k binds to Ddx6 and brings it along microtubules to aggresomes, where it is likely inactivated. The removal of Ddx6 from p bodies may then result in the dispersal of Pat1b leading to a larger number of finer cytoplasmic foci. Some of these dispersed Pat1b foci were still seen to colocalize with Ddx6, but there were also many that did not colocalize and were distinct from Ddx6 foci. With E4 11k from other serotypes, direct binding to Ddx6 and colocalization in aggresomes has not been observed, however, Ddx6 has been shown to be disrupted (Greer et al. 2011). The lack of observable Pat1b disruption with Ad9 and to a certain degree,

Ad12, suggests that there may be something else going on to cause the dispersal of Pat1b in Ad5-infected cells.

It is not clear at this time whether these smaller foci of Pat1b are still functional in their ability to regulate mRNA degradation. As they appear to be aggregates of Pat1b protein, it is likely that they are ribonucleoprotein aggregates as Pat1b acts as a scaffolding protein. However, the full complement of proteins necessary for mRNA degradation may not be present in each of the smaller, dispersed foci. It is also possible that these foci represent a separate, distinct subcellular component of the cell. We do know that the reorganization is dependent on E4 11k and that E4 11k binds to Ddx6. While we have not performed a co-immunoprecipitation of E4 11k and Pat1b and thus do not know if these two proteins bind to each other, it is unlikely given that Pat1b does not localize to aggresomes with E4 11k during an infection. It could be possible that the binding of E4 11k to Ddx6 disrupts the binding of Ddx6 to Pat1b. We have shown that Pat1b does not appear to respond to infection or chemical stress in the same way as other p body proteins. Increasing in foci number while decreasing in size in response to E4 11k expression has not been seen before and the lack of relocalization to aggresomes during infection or chemical stress is unique as well.

One hypothesis as to the function of this disruption of p bodies has been to control late gene expression during an infection. It is important for viruses to be able to prioritize translation of their late viral mRNAs over the cellular mRNAs. Viruses often have multiple mechanisms to be able to achieve the shutdown of host protein synthesis and promote the synthesis of late viral structural proteins. Both E1b 55k and E4 11k were shown to have roles in the regulation of late gene expression by looking at newly synthesized proteins in mock-infected cells, and wild-type and several deletion virus-infected cells (Shepard and Ornelles 2004). There was a wide array of proteins being expressed in mock-infected cells but contrastingly, by 30 hours post wild-type virus infection, there were only a few proteins being expressed. These few proteins being expressed, however, were at high levels and aligned with the sizes of several major late viral structural proteins. In each single mutant, E1b 55k-deleted or E4 11k-deleted, there was a slight change from wild-type, with less late viral protein and more cellular proteins being translated. In the double mutant that was deleted in both E1b 55k and E4 11k, the protein expression levels were much more similar to mock-infected cells (Shepard and Ornelles 2004). The mechanism by which either protein contributes to the regulation of late viral gene expression is unknown. When a connection was made between E4 11k and p bodies, cellular structures that help to regulate mRNA metabolism, it was hypothesized that this interaction facilitated the prioritization of translation of late viral mRNAs.

Interestingly, another function of E4 11k is to disrupt another cellular body, the PML nuclear bodies (Doucas et al. 1996). These two cellular bodies are similar in that they are membraneless organelles that play a role in gene regulation. Their formation is based on liquid-liquid phase separation and while it is not entirely clear how the viral protein achieves this disruption, the oligomerization of E4 11k appears to be critical for the disruption of PML nuclear bodies (Patsalo et al. 2012). E4 11k has been shown to bind to some TRIM proteins (TIF1alpha and TIF1gamma) via the coiled coil domains (Vink et al. 2015), however, Ddx6 does not have coiled coil domains so there must be another domain that it binds to. Previous work has demonstrated that E4 11k binds to both the N- and C-terminal RecA-like domains of Ddx6 (Sharan 2012). It has previously been shown that Ddx6 binds to Pat1b through its C-terminal RecA-like domain (Ozgur and Stoecklin

2013). It was also suggested that this Ddx6-Pat1b binding leads to further aggregation with other p body proteins leading to p body formation. If E4 11k disrupts the binding of Ddx6 and Pat1b, it could explain the dispersal of Pat1b into finer foci. Further research would need to be performed to narrow down the binding requirements of E4 11k and Ddx6, which can then lead to studies to determine if binding to Ddx6 is required for the relocalization of other p body proteins.

Further exploration of this disruption of p bodies during an infection may provide support for the role of p bodies in late gene expression hypothesis. In the future, we would like to look at Pat1b during late times of an infection with more serotypes. Since there were still some significant differences in the number of Pat1b foci with Ad12 E4 11k expression but no change with Ad9, we would like to see if there are other differences with serotypes Ad3 and Ad4. We would also like to observe protein levels of several p body proteins during an adenovirus infection to make sure their protein levels are not contributing to the phenotypes observed. Additionally, we would like to observe cellular protein expression levels late during infection when the interaction between Ad5 E4 11k and Ddx6 is disrupted. It may also be useful to observe newly synthesized proteins when Ddx6 and/or Pat1b has been knocked down. The role of p bodies in cellular biology is not well elucidated either, so these studies will not only contribute to our understanding of their role during dsDNA virus infections, but also how that role may contribute to the efficiency of gene expression in healthy cells.

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