2013

Evading Innate and Adaptive Immunity During Adenovirus Cell Entry

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EVADING INNATE AND ADAPTIVE IMMUNITY DURING ADENOVIRUS CELL ENTRY

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

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CHICAGO, IL

MAY 2013
ACKNOWLEDGMENTS

I would like to thank my wonderful mentor, Dr. Chris Wiethoff, for making this dissertation possible. I cannot thank him enough for taking me in, guiding me through this process, his scientific insight and ideas, all while providing a supportive environment. I also thank my committee chair, Dr. Tom Gallagher, for all his guidance and ideas, and my committee members Dr. Ed Campbell, Dr. Phong Le and Dr. Adriano Marchese for helping me get to this point. I thank the Department of Microbiology and Immunology faculty, staff and students for their time and help. I’d especially like to thank Dr. Ed Campbell for the copious amount of microscope time he allowed me to have.

I also want to thank my family-Mom and Dad for their support and to my sister (and friend) Lianne for her love and for helping me upgrade my wardrobe.

Most importantly, I thank my friends for all their support. I especially thank my year of classmates-Bruno, Justin and Andy for their love, support, sense of humor and dancing skills, and Anita for being my sports buddy and for always letting me be me. I also thank Mariko for introducing me to Sushi Station and my lab mate, and fellow Aquarian, Katie for being the best lab buddy I could ask for. There are so many others to thank-Bridget, Jay, Linda, Nestor (and Lucy), Val, Allison, Eddie, Arlene, Jodie, Tiff and people that I’m probably forgetting. I never could have done this without you.
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ABSTRACT

Adenovirus (Ad), a non-enveloped, dsDNA virus, enters cells via clathrin-mediated endocytosis. For delivery of the viral genome to the nucleus, Ad must penetrate the endosomal membranes. This membrane penetration event is relatively catastrophic, creating membrane defects sufficient for the passage of a 90 nm diameter capsid across cell membranes. This membrane penetration event can be sensed as a danger signal by host cells leading to a proinflammatory response. Recent observations suggest that adenovirus type 5 (Ad5) capsid uncoating occurs at the cell surface upon binding to both the coxsackievirus and adenovirus receptor and αv integrins. This uncoating event leads to the exposure of the capsid membrane lytic protein VI. Using the cytosolic protein galectin-3 (gal3) as a marker of membrane rupture, we demonstrate that Ad5 rupture of membranes occurs at or near the cell surface upon exposure of protein VI. However, translocation of the virus across ruptured membranes occurs more frequently from perinuclear endosomal locations. Trafficking of Ad5 in gal3+ vesicles is in agreement with differing sites of membrane rupture and endosomal egress. Additionally, while Ad5 rupture of cell membranes is unaffected by microtubule depolymerization, egress of Ad5 virions from ruptured endosomes is dependent upon microtubules.

Ad pVI encodes a highly conserved PPxY motif, capable of recruiting Nedd4-family E3 ubiquitin ligases. Mutation of the pVI-PPxY motif (Ad-WT) to PGAA (Ad-M1) does not impair virus endocytosis or endosomal membrane rupture but results in reduced specific
infectivity. We show that microtubule-dependent egress of Ad5 capsids from ruptured membranes is mediated via the conserved PPxY motif. Ad5-M1 colocalizes with gal3 significantly more than Ad5-WT, and while microtubule depolymerization increased Ad5-WT colocalization with gal3, Ad5-M1 colocalization was unaffected.

Further, the defect in Ad-M1 nuclear trafficking results from sequestration of the virus into autophagosomes. Although both Ad-WT and Ad-M1 infection induce autophagosome formation, a greater percentage of these autophagosomes contain Ad-M1 compared to Ad-WT. Further, Ad-M1 virus colocalizes with the lysosome marker LAMP1 more than Ad-WT several hours after infection. Ad-M1 colocalization with LAMP-1 is reduced and infectivity is restored to Ad-WT levels when autophagy is inhibited by RNAi silencing of Atg5 expression. Additionally, RNAi silencing of galectin-8 (gal8), which is also recruited to sites of Ad5 membrane rupture, restored Ad5-M1 infectivity to Ad5-WT levels and decreased Ad5-M1 colocalization with the autophagosome marker LC3 and LAMP1 to Ad5-WT levels. Further, activation of T cells to Ad5 capsid antigens was increased with Ad5-M1 transduction compared to Ad5-WT. These data demonstrate that a conserved PPxY motif in Adenovirus pVI is important for Ad5 endosome escape to evade autophagy during entry.
CHAPTER I
INTRODUCTION

Adenoviruses

Adenoviruses (Ads) are non-enveloped, double-stranded DNA viruses that were isolated over fifty years ago and cause upper respiratory tract, gastrointestinal tract and ocular infections (Hilleman and Werner, 1954; Rowe, 1955). Approximately 40 proteins are encoded in the Ad genome, 12 of which are present in the assembled virion (Figure 1) (Nemerow et al., 2009; Russell, 2009). There are over 60 human Ad serotypes, which are classified into 7 subgroups based on hemagglutination properties and sequence homology (Benko and Harrach, 2003; Aoki et al., 2011; Seto et al., 2011; Matsushima et al., 2012). Although acute adenoviral infections are generally self-limiting in healthy individuals, Ad infections can cause severe complications in immunocompromised patients (Hierholzer, 1992; Bordigoni et al., 2001; Leen and Rooney, 2005; Hoffman, 2006; Gray et al., 2007). However, Ads can still cause significant respiratory disease in healthy individuals. For example, healthy individuals with Ad serotype 14 infections resulted in hospitalization rates of almost 40% with a 5% mortality rate (Anonymous, 2007). Additionally, they cause severe pneumonia in military recruits (Gray et al., 2007; Kajon et al., 2007). Since infections with many Ad serotypes are self-limiting, Ad-based vectors are currently explored as gene therapy and vaccine vectors. Ad gene therapy vectors have been explored for a number of applications, including cancer and
Figure 1. Adenovirus capsid structure. Diagram showing the 7 capsid proteins plus the L3-23K protease. Protein IIIa, protein VI and protein VIII are located in the interior of the capsid. Also contained in the capsid is the L3-23K protease/AVP. Not shown are the Terminal protein, Protein Mu, Protein VII, and Protein V, where are all associated with the viral DNA (also not shown). Adapted from (Nemerow et al., 2009).
cardiovascular disease, but uses are limited due to the potent inflammatory response elicited by Ad (Harvey et al., 2002a; Raper et al., 2003; Muruve, 2004; Henry et al., 2007; Rissanen and Yla-Herttuala, 2007; Shirakawa, 2008). This inflammatory response, however, make Ads attractive candidates as vaccine vectors and have been explored for a variety of pathogens, including HIV, Ebolavirus and influenza (Hoelscher et al., 2008; Priddy et al., 2008; Lasaro and Ertl, 2009; Geisbert et al., 2011). The current generation of Ad-based vaccines can exhibit high immunogenicity, but often lack efficacy. For example, human Ad type 5 (Ad5) is often used as the vaccine vector, but most people already have neutralizing antibodies to Ad5, limiting their effectiveness (Lasaro and Ertl, 2009). Additionally, Ad vectors are poor inducers of CD4+ T cells, important for generating an antibody response. Recent studies with Ad-based vaccine vectors focus on improving the CD4+ T cell and antibody responses (Parks, 2005; Bayer et al., 2010; Matthews, 2011). Additionally, Ad-based vectors can generate such high levels of antigen that CD8+ T cells are of the effector memory type, instead of central memory (Yang et al., 2006). Central memory CD8+ T cells are considered the protective memory population (Wherry et al., 2003). They exhibit greater basal homeostatic proliferation and upon antigen exposure, mount a larger magnitude of expansion compared to effector memory CD8+ T cells (Wherry et al., 2003). Additionally central memory cells confer more protection against viral and bacterial rechallenge (Wherry et al., 2003).

**Adenovirus Cell Entry**

**Adenovirus Receptors**

To initiate infection, human Ads bind to cells via a high affinity interaction
between the knob domain of the Ad fiber protein and a primary attachment receptor
B and Adenovirus Receptor (CAR) as their primary attachment receptor (Freimuth et al.,
1999). Subgroups C and D also use CAR in addition to other receptors (subgroup C can
also use heparan sulfate proteoglycans (HSPG), MHC-I and VCAM-I and subgroup D
viruses can also use sialic acid and CD46) (Bergelson et al., 1997; Hong et al., 1997;
Freimuth et al., 1999; Huang et al., 1999; Arnberg et al., 2000; Chu et al., 2001;
Dechecchi et al., 2001). Subgroup B viruses use CD46, HSPG, CD80/86, and
Demsoglein 2 (Gaggar et al., 2003; Sharma et al., 2009; Wang et al., 2011).
Additionally, more recent studies suggest the cell attachment during systemic infection
may rely on the association of soluble serum cofactors with the capsid (Parks, 2005;
Shayakhmetov et al., 2005; Alba et al., 2009).
Although the fiber-receptor association mediates Ad attachment to cells, it does
not foster rapid uptake of the virus into cells. Instead, a second interaction between the
Arginine-Glycine-Aspartate (RGD) motif of the Ad penton base and αv integrins
mediates Ad cell uptake, activating cell signaling molecules required for clathrin-
mediated endocytosis (Li et al., 1998a; Li et al., 1998b; Nemerow and Stewart, 1999;
Smith et al., 2010b). Ad trafficking within endosomal compartments appears to depend
upon the nature of Ad-receptor interactions, with subgroup C serotypes trafficking to
early endosomal compartments prior to endosomal escape (Gastaldelli et al., 2008) while
subgroup B viruses traffic to late endosomes/lysosomes prior to endosomal escape
(Miyazawa et al., 2001; Shayakhmetov et al., 2003).
**Adenovirus Uncoating**

After Ad attachment, Ad infection requires viral uncoating. After engagement with its receptor, the Ad fiber is shed at the cell surface (Nakano et al., 2000). Recent studies demonstrate that Ad fiber binding to CAR initiates movement within membranes (or drifts), while penton base-integrin associations are spatially more confined (Burckhardt et al., 2011). These CAR-mediated drifts with integrin binding supported fiber shedding from Ad particles (Burckhardt et al., 2011).

While shedding of the fiber protein is independent of endocytosis, Ad internalization likely initiates further stepwise disassembly of the adenovirus capsid, or viral uncoating (Greber et al., 1993). Early biochemical studies suggested that capsid uncoating requires exposure to low pH for release of internal capsid proteins and some hexon (Seth et al., 1984, 1985b; Greber et al., 1993; Prchla et al., 1995). However, further studies indicate that low pH may not be a requirement for many serotypes of Ads. Instead, Adenovirus trafficking within endosomal compartments appears to depend upon the nature of Ad-receptor interactions, with subgroup C adenovirus serotypes trafficking to early endosomal compartments prior to endosomal escape (Gastaldelli et al., 2008) while subgroup B viruses traffic to late endosomes/lysosomes, where Ad would encounter low pH (Miyazawa et al., 2001; Shayakhmetov et al., 2003). The presence of weak bases, such as ammonium chloride, does not affect Subgroup C viral uncoating (Rodriguez and Everitt, 1996).

**Adenovirus Membrane Rupture**

Endosomal membrane rupture and virion endosomal escape requires viral
uncoating. Initial studies supporting a role for viral uncoating and endosome lysis were obtained using a temperature sensitive mutant of Ad2 (ts1-39), which, when propagated at the non-permissive temperature, fails to uncoat (Weber, 1976; Mirza and Weber, 1979). This virus contains a point mutation (P137L) in the L3-23K viral protease, leading to decreased protease incorporation into the virion (Weber, 1976; Rancourt et al., 1995). The L3-23K protease functions within the virion to cleave six different Ad structural proteins in the immature virus into their mature form (Webster et al., 1989; McGrath et al., 1996; Silvestry et al., 2009). An immature virus has increased capsid stability (Silvestry et al., 2009). During infection, this virus fails to uncoat, rupture membranes and escape the endosome, and is degraded through late endosomes/lysosomes (Greber et al., 1996; Gastaldelli et al., 2008).

Later studies confirmed the link between capsid disassembly and endosomal lysis, showing that viral uncoating releases an internal capsid protein, protein VI (pVI), which was recently shown to rupture endosomal membranes, required for the partially disassembled virion to enter the cytoplasm (Wiethoff et al., 2005; Maier et al., 2010; Moyer et al., 2011). Antibodies to pVI do not stain the viral capsid prior to endocytosis (Wodrich et al., 2010; Burckhardt et al., 2011). However, within 5 minutes of viral internalization, pVI could be detected by (Wodrich et al., 2010). Further supporting the notion that subgroup C viruses do not require low pH to uncoat, most of the pVI detected at 5 minutes costained with viral particles that were near the cell periphery (Wodrich et al., 2010). Additional studies indicate that pVI exposure is linked to viral-receptor interactions at the cellular surface (Burckhardt et al., 2011). Infection of CAR-deficient
L929 cells with Ad5-RGD4C, which can bind to integrins without binding CAR, showed less pVI exposure compared to infection of L929 cells that do express CAR, while endocytosis levels were the same between the two cell types (Burckhardt et al., 2011).

PVI, a structural protein present as ~342 copies in the adenovirus virion, possesses a number of functional properties during the Ad life cycle. This protein is suggested to be a trimer of dimers located within the cavity of all hexon trimers in the virion (Stewart et al., 1993; Saban et al., 2006). PVI was recently found to possess all of the in vitro membrane disrupting activity of the adenovirus capsid and antibody neutralization was recently shown to prevent adenovirus membrane rupture (Wiethoff et al., 2005; Maier et al., 2010). Although there is no crystal structure for pVI, it is predicted to be a four-helix bundle at the N-terminal end, with the rest of the protein mainly unstructured (Maier et al., 2010). The N-terminal four-helix bundle posses all the in vitro membrane lytic activity (Wiethoff et al., 2005; Maier et al., 2010). The first alpha-helix in the four-helix bundle is predicted to be amphipathic (Wiethoff et al., 2005). This predicted amphipathic alpha helix increases the membrane affinity of pVI and exhibits the same membrane rupturing ability as the entire pVI, although a pVI that does not contain the first helix still exhibits some membrane lytic activity (Wiethoff et al., 2005; Maier et al., 2010). Genetic deletion of the amphipathic alpha helix or specific mutations within the helix decrease pVI membrane affinity and membrane rupture (Wiethoff et al., 2005; Maier et al., 2010; Moyer et al., 2011).

Using an established distance-dependent quenching of tryptophan fluorescence by bromine atoms method, Maier et al. determined the depth within the lipid of conserved
tryptophan residues within the pVI amphipathic alpha helix as well as additional residues within the subsequent three alpha helices (Maier et al., 2010; Maier and Wiethoff, 2010). From the data they proposed that the four alpha helices lie parallel to the membrane (Maier et al., 2010; Maier and Wiethoff, 2010). Maier et al. next demonstrated that pVI membrane binding fragments membranes by inducing positive membrane curvature stress (Maier et al., 2010). These in vitro observations of the mechanism of pVI membrane rupture are supported by the observation that Ad infection facilitates cytosolic translocation of co-endocytosed high molecular weight molecules, such as 70kDa dextrans or whole parvoviruses, in cell culture (Prchla et al., 1995; Farr et al., 2005). This suggests that Ad rupture of endosomes is catastrophic, releasing Ads and endosomal contents into the cytoplasm.

Microtubules Facilitate Adenovirus Cell Entry

Viruses, including Ads, must traffic long distances through the cytoplasm to the nucleus following entry. One possible way to accomplish this task is by diffusion of the virus through the cytoplasm. However, the cytoplasm is considered an extremely crowded medium, because organelles and cytoskeletal components pose as barriers, limiting free viral movement (Luby-Phelps, 2000; Leopold and Pfister, 2006). Studies using fluorescence recovery after photobleaching suggest that dextran particles greater than 20nm are virtually immobile in the cytoplasm (Luby-Phelps, 2000; Leopold and Pfister, 2006). Therefore a 90nm Ad particle to freely diffuse throughout the cytosol to get to the nucleus would not be an efficient mechanism for viral infection.

Instead, many viruses trafficking to the nucleus by using microtubule motors
(Dohner et al., 2005). Microtubules are part of the cytoskeleton that help the cell maintain its shape and have multiple cellular functions (Desai and Mitchison, 1997). While microtubules can generate movement due to their constant assembly and disassembly, they can also serve as scaffolds for microtubule-associated proteins (or MAPs), which include motor proteins, to move on (Desai and Mitchison, 1997). The two major microtubule motors are dynein and kinesin. The protein complex cytoplasmic dynein 1 contains six different subunits. There are two heavy chains (DYNC1H1), each containing a globular motor domain and an N-terminal stalk. The N-terminal stalk interacts with dimers of intermediate chains and light intermediate chains (DYNC1LI and DYNC11I). Three light chain families bind the intermediate chains, and collectively, make up the cargo binding domain (Leopold and Pfister, 2006). In addition to direct binding to the dynein protein complex, binding can also be mediated through a second protein protein complex, dynactin (Vaughan, 2005). For example, the p150 subunit of dynactin binds to the dynein intermediate light chain and is linked to membranous organelles through actin-related protein 1 (Arp1) (Vaughan, 2005).

In general, dynein transports cargo to the minus end of microtubules, often anchored at the microtubule organization center (MTOC), while kinesin transports cargo to the plus end of microtubules, which are often located in the cell periphery (Hook and Vallee, 2006; Hirokawa et al., 2009). Kinesin and dynein motors are capable of generating instantaneous velocities (or short bursts of movement) of 2μm per second (Leopold and Pfister, 2006). Since the average diameter of a cell is 20-40μm, a virus should be able to reach the nucleus in less than one minute traveling via microtubules
(Leopold and Pfister, 2006). While recent reports indicate a number of viruses using dynein to traffic to the nucleus, including herpes simplex virus (HSV1) and human immunodeficiency virus type 1 (HIV1), how viruses specifically recruit microtubules and when these components are recruited is poorly understood (Dodding and Way, 2011).

Initial evidence that linked Ads with microtubules comes from electron microscopy studies that show Ad particles in close association with microtubules during infection (Dales and Chardonnet, 1973; Miles et al., 1980). Subsequent studies linked Ad trafficking with microtubules by depolymerizing microtubules with pharmacologic agents, such as nocodazole (NOC). These studies observed decreased Ad perinuclear accumulation or infectivity in NOC-treated cells (Miles et al., 1980; Everitt et al., 1990; Suomalainen et al., 1999; Leopold et al., 2000; Mabit et al., 2002). Additionally, overexpression of the dynactin subunit dynamitin or microinjection of anti-dynein prevents Ad translocation to the nucleus (Suomalainen et al., 1999; Leopold et al., 2000). Subsequent studies showed that MAPs, specifically dynein, increased Ad interaction with microtubules (Kelkar et al., 2004). Collectively these studies demonstrate an involvement for microtubules and MAPs for Ad infection.

Several groups have since suggested that determinants in the major Ad capsid protein, hexon, and pVI (described later in detail) influence microtubule-dependent trafficking of adenovirus virions in the cytoplasm (Smith et al., 2008; Bremner et al., 2009; Wodrich et al., 2010; Strunze et al., 2011). Smith et al. showed that a hexon-specific neutralizing antibody (9C12) decreased Ad5 MTOC accumulation, even though the antibody increased Ad5 microtubule binding (Smith et al., 2008). Bremner et al.
found that Ad5 particles colocalized with dynein heavy, intermediate and light intermediate chains and dynactin subunits (Bremner et al., 2009). By purifying hexon from late stage-infected cells and performing pull downs with dynein components in vitro, they found that the hexon pulled down both intermediate and light intermediate chains 1 and 2 (Bremner et al., 2009). These associations were increased upon hexon exposure to low pH (Bremner et al., 2009). Although not extensively studied, Ad engagement of microtubules has been thought to occur after endosome escape, since one previous study observed moving Ad capsids were at neutral pH, and not acidic endosome compartments (Leopold et al., 2000). Since increasing evidence points to Ad5 not needing or encountering low pH during endocytic entry, further studies are needed to determine if a hexon-dynein interaction occurs in vivo.

After Ad accumulation at the MTOC, the virus must then make its way to the nucleus for genome delivery. Ad2/5 nuclear envelope accumulation requires the nuclear export factor CRM1 (Strunze et al., 2005). Pharmacological inhibitors or siRNA knockdown of CRM1 arrested Ad2/5 in the cytosol or at the MTOC and decreased genome delivery (Strunze et al., 2005).

Docking of partially disassembled adenovirus virions at the nuclear envelope occurs at nuclear pore complexes. While Ad particles are seen around the entire periphery of the nuclear envelope in cell lines, Ad particles accumulate at the part of the nuclear envelope closest to the MTOC in primary cells (Leopold et al., 1998; Bailey et al., 2003). Recently, Strunze et al. demonstrated that kinesin binds to the Ad capsid protein IX and to several proteins in the nuclear pore complex for further capsid
disassembly, aiding in Ad DNA genome delivery to the nucleus (Strunze et al., 2011). Adenovirus that does not contain pIX in the capsid exhibits a 2-3-fold defect in specific infectivity compared to its wild type counterpart (Strunze et al., 2011 and data not shown). Ads added to HeLa cells that have been knocked down for the kinesin components kinesin light chain 1 (KLC1) or Kif5C exhibit a 2-fold defect in specific infectivity compared to control siRNA treated cells (Strunze et al., 2011). Roles for nuclear filament protein Nup214, as well as soluble factors, hsc70 and histone H1 have been implicated in nuclear import of the viral genomic DNA (Saphire et al., 2000; Trotman et al., 2001; Strunze et al., 2011).

**Ubiquitination**

Ubiquitin plays essential roles in multiple cellular processes. Ubiquitin is conjugated to proteins via amide formation between the ubiquitin C-terminus and ε-amino groups on lysine side chains of proteins (Kerscher et al., 2006). Conjugation of ubiquitin to proteins relies on the activity of three classes of enzymes termed E1, E2, and E3-ubiquitin ligases. The E1 activating enzyme forms a thiolester bond with ubiquitin. The ubiquitin is subsequently transferred onto the E2 ubiquitin conjugating enzyme. The ubiquitin moiety is finally transferred onto a lysine side on the target protein by the E2 and E3 ubiquitin ligase.

Once a ubiquitin moiety is appended to a given protein, subsequent ubiquitin molecules can be either conjugated to another lysine residue of the same protein (multiubiquitination) or appended to one of seven lysine residues on ubiquitin itself (polyubiquitination) (Kim and Rao, 2006). While protein ubiquitination was initially
studied for its role targeting proteins for proteasomal degradation via appendage of K48 linked polyubiquitin chains to target proteins, more recent work has highlighted a role for mono- or multi-ubiquitination in protein trafficking and activity and the assembly of signaling complexes (Kim and Rao, 2006).

Protein ubiquitination is highly dynamic, with possibly hundreds of E3 ubiquitin ligases and approximately 100 deubiquitinases (Nijman et al., 2005) tightly regulating ubiquitin modifications of proteins. It is the E3-ubiquitin ligase which provides the substrate specificity and ultimately conjugates ubiquitin to the target protein (Nagy and Dikic, 2010). Ubiquitin E3 ligases are classified into three types based on their catalytic domains. The Homologous to E6-associated protein C-Terminus (or HECT) contains a catalytic cysteine residue that accepts and transfers ubiquitin from the E2 to the target protein (Li and Ye, 2008). The Really Interesting New Gene (or RING) finger and the U box E3 ligases bring the E2 and target protein in close proximity to each other to allow for ubiquitin transfer, instead of bonding to ubiquitin like the HECT family of E3-ubiquitin ligases (Hatakeyama and Nakayama, 2003; Fang and Weissman, 2004; Li and Ye, 2008).

One of the most well studied members of the HECT family of E3-ubiquitin ligases are members of the Nedd4 family. These ligases contain an N-terminal phospholipid-binding C2 domain, 1-4 WW domains and a C-terminal HECT domain (Shearwin-Whyatt et al., 2004). The C2 domain mediates membrane association, while the WW domains recognize motifs to mediate protein-protein interactions (Shearwin-Whyatt et al., 2004). WW domains recognize poly-proline motifs, phosphorylated
serines or threonines, or PPxY motifs, where P is proline, Y is tyrosine and x is any amino acid (Sudol et al., 2005). These motifs can be within the protein that will be ubiquitinated, or on an adapter protein that recruits the E3-ubiquitin ligase to the target protein (Sudol, 1996; Harty et al., 2000; Sudol et al., 2005; Sullivan et al., 2007).

Many examples have highlighted the influence of the ubiquitin/proteasome system (UPS) on viral replication and egress such as in DNA virus replication in the nucleus (Weitzman et al., 2011) or the budding of enveloped viruses (Bieniasz, 2006). Much less is currently known about the contributions of the UPS during viral entry.

Effect of Ubiquitination During Viral Cell Entry

Evidence for ubiquitination during viral cell entry is limited. Two studies provide evidence for UPS during murine coronavirus (MHV) cell entry. The first study depleted the free ubiquitin pool using the proteasome inhibitors lactacystin and MG132. These inhibitors did not inhibit MHV internalization, but did decrease viral nucleocapsid protein expression (Yu and Lai, 2005). A later study confirmed the finding generating a temperature-sensitive E1 cell line (Raaben et al., 2010). When shifted to the non-permissive temperature, the E1 ubiquitin ligase is inactivated, preventing ubiquitination of proteins (Raaben et al., 2010). They also found no difference in particle internalization, but did observe decreased MHV RNA synthesis when cells were shifted to the non-permissive temperature (Raaben et al., 2010). To further characterize the defect in MHV infection, Yu and Lai used centrifugation to separate early and late endosome fractions and lysosomes (Yu and Lai, 2005). MHV was found in fractions where endosomes and lysosomes are found in MG132 treated cells, compared to the virus
found in cellular fractions not associated with endosome/lysosome compartments in untreated cells (Yu and Lai, 2005). Additionally, the virus from MG132-treated cells was RNase resistant in the endosome/lysosome fractions, whereas the fractions that contained virus isolated from untreated cells was RNase sensitive, suggesting that the viral particles were in tact and were contained within the endosome/lysosomes (Yu and Lai, 2005).

However, Yu and Lai could not detect any MHV ubiquitinated proteins during entry, so how the UPS facilitates MHV cell entry is yet to be determined.

Studies from Khor et al. used MG132 treatment to demonstrate the importance of the UPS during influenza infection (Khor et al., 2003). Compared to untreated cells, there was decreased NP expression during influenza infection in cells treated with MG132 (Khor et al., 2003). No difference in expression was observed when MG132 was added after two hours of infection, a time prior to viral replication, suggesting the defect was in the viral entry process (Khor et al., 2003). Immunofluorescence microscopy studies determined that the virus colocalized with markers of late endosomes or sorting and recycling endosomes during MG132 treatment, compared to nuclear localization in untreated cells (Khor et al., 2003). These data suggest that influenza virus requires the UPS for cell entry, possibly by affecting endosomal sorting.

Effect of Ubiquitination During Adenovirus Cell Entry

ADENOVIRUS CAPSID PROTEINS AND UBIQUITINATION

Protein VI

Recently, evidence for direct engagement of the Ad5 capsid of the UPS during cell entry has been suggested (Wodrich et al., 2010). Wodrich et al. reported the
identification of a highly conserved PPxY motif in capsid protein VI (Wodrich et al., 2010). This motif is present within all sequenced human adenoviruses (Wodrich et al., 2010). Wodrich et al. demonstrated that although there was no defect in endosomal membrane rupture by recombinant Ad5 in which the pVI PPSY motif was mutated to PGAA (M1), there was a substantial defect in cytosolic trafficking of the M1 mutant virus to microtubule organizing centers (MTOC) and subsequent nuclear accumulation (Wodrich et al., 2010). In vitro studies demonstrated that protein VI is oligo-ubiquitinated (2-3 ubiquitins) by a cytosolic factor and that this ubiquitination is abrogated upon mutation of the PPSY motif in Ad5 protein VI to PGAA (Wodrich et al., 2010). When overexpressed in U2OS cells, Ad5 protein VI containing the M1 mutation failed to associate with members of the Nedd4 family of E3 ubiquitin ligases, Nedd4.1, Nedd4.2, AIP4, WWP1 and WWP2 as assessed by co-precipitation or fluorescence colocalization (Wodrich et al., 2010). Knockdown of Nedd4.2, but not Nedd4.1, AIP4 or other Nedd4 family members by siRNA lead to a reduction in Ad5 transduction efficiency as well as reduced colocalization of Ad5 with MTOCs (Wodrich et al., 2010). Further, live cell imaging of the intracellular trafficking of protein VI demonstrated that it was highly mobile, apparently moving in association with vesicular structures in a microtubule dependent manner (Wodrich et al., 2010). Together, these data lead Wodrich et al. to propose that protein VI facilitates the microtubule dependent trafficking of adenovirus capsids toward the MTOC, ultimately leading to nuclear delivery of the viral genome. The exact role for the protein PPxY in adenovirus cell entry remains to be elucidated. Given that the PPxY in protein VI is so highly conserved amongst adenovirus
serotypes, it is interesting to consider whether the fixation of this motif in the adenovirus genome resulted from a strict requirement for microtubule dependent movement of the virus within the cytoplasm, or whether it may have evolved to evade host restriction factors which prevented “PPxY-less” adenoviruses from efficiently delivering their genomes to the nucleus for viral replication. Given several reports which indicate that certain cell types support microtubule independent infection by adenoviruses, the latter possibility warrants further exploration.

**Penton Base**

The adenovirus penton base is a homopentamer present at the vertices of the adenovirus capsid. In addition to anchoring the receptor binding fiber protein to the surface of the adenovirus capsid, binding of the penton base to \( \alpha_v \) integrins through a conserved RGD motif initiates viral endocytosis (Nemerow and Stewart, 1999; Smith et al., 2010b). The adenovirus penton base contains two PPxY motifs in its N-terminal domain, also conserved across all known human serotypes (Galinier et al., 2002). *In vitro* and *in vivo* studies by Galinier et al. demonstrate that the Ad2 and Ad3 penton bases also binds to WW-domain containing proteins, with the co-chaperone protein Bcl-2-associated athanogene 3, BAG3, identified as a penton base interacting protein in a yeast two hybrid screen (Galinier et al., 2002; Gout et al., 2010). Galinier et al. further demonstrated that members of the Nedd4 family could also bind penton base via the N-terminal PPxY motif (Galinier et al., 2002). Although Galinier et al. determined that Ad2 penton binding to the Nedd4 family member WWP1 formed a stable complex, they did not determine if the penton base itself was ubiquitinated, nor if deletion of the first
PPxY motif affected adenovirus cell entry. Wodrich et al. later determined that the penton base was ubiquitinated, but did not test if lack of ubiquitination affected Ad cell entry (Wodrich et al., 2010).

More recently, Gout et al. demonstrated that BAG3 interactions with penton base occur within infected cells as determined by co-immunoprecipitation (Gout et al., 2010). Further, penton base overexpression in cell culture, recruits BAG3 to the nucleus in a PPxY-dependent manner (Gout et al., 2010). These experimental results suggest that BAG3 may be important for adenovirus infection. To examine this possibility Gout et al. demonstrated that siRNA knockdown of BAG3 attenuated transgene delivery by replication-incompetent Ad5 vectors based on a reduction in luciferase expression (Gout et al., 2010). They also showed by single round infectivity assays that less progeny virions were produced in BAG3-knockdown cells (Gout et al., 2010). This study did not demonstrate the effect of mutating the penton base PPxY motifs on viral cell entry or replication. Nonetheless, these data suggest that BAG3 plays a role in adenovirus cell entry. As with the PPxY motifs in protein VI, further study of the penton base PPxY motifs is warranted to elucidate their role in adenovirus cell entry.

*L3-23K Protease*

Adenovirus virions contain approximately 11 copies of the L3-23K cysteine protease (or Avp) (Greber et al., 1996). Avp has a well defined role in adenovirus virion assembly by proteolytically processing adenovirus capsid preproteins during viral maturation, presumably to lock the capsid into a metastable state necessary for subsequent entry of these newly formed virions into neighboring, uninfected cells
Efficient adenovirus cell entry requires Avp’s protease activity (Cotten and Weber, 1995; Greber et al., 1996). Inhibition of the protease activity via reduction and alkylation of the active site cysteine or oxidation of the active site cysteine by copper chloride treatment reduced adenovirus transgene expression (Cotten and Weber, 1995; Greber et al., 1996). In one report, the defect in cell entry of protease-inactivated virions was found to occur after the virus had escaped from endosomes into the cytoplasm but prior to docking of the virus at the nuclear envelope (Greber et al., 1996). The function of Avp during the cell entry process has previously been ascribed to further proteolytic processing of the capsid, although this phenomenon has not been completely characterized. Interestingly, Avp possesses deubiquitinating and deISGylating activity, which has been observed in vitro and in vivo (Balakirev et al., 2002). While deubiquitinating activity was observed after 12 hrs during infection based on global reduction in the number of ubiquitinated proteins observed in western blots, the deubiquitinating activity of Avp was not examined at earlier times during cell entry (Balakirev et al., 2002). Given the involvement of Avp in adenovirus cell entry, it is interesting to consider whether efficient intracellular trafficking to the nucleus requires Avp deubiquitination of one or several host or viral proteins during adenovirus entry.

**UBIQUITINATION AND ADENOVIRUS RECEPTORS**

One potential mechanism influencing adenovirus receptor biology is the UPS. Previous reports demonstrate receptor interactions with UPS machinery during pathogen entry (Kohr et al., 2003; Abrami et al., 2006; Coller et al., 2009). Tumor endothelial marker 8 (TEM8), a receptor for *Bacillus anthracis* lethal toxin, is ubiquitinated within
10 minutes of toxin treatment (Abrami et al., 2006). Mutating one of the 16 lysine residues greatly diminished TEM8 ubiquitination, which lead to a delay in TEM8 endocytosis in response to toxin treatment, indicating that ubiquitination of TEM8 is necessary for efficient endocytosis (Abrami et al., 2006). TEM8 ubiquitination (and subsequent internalization) depended on the Cbl E3 ubiquitin ligase, as knockdown of Cbl abolished toxin-induced TEM8 ubiquitination and internalization (Abrami et al., 2006). HCV colocalizes with Cbl prior to internalization, though whether Cbl is required for HCV internalization was not determined (Coller et al., 2009). Thus, pathogen receptor ubiquitination may influence internalization, though whether ubiquitination of Adenovirus receptors influences Ad cell entry has not directly been tested.

Additionally, influenza virus requires ESCRT machinery for transit of viral ribonucleoproteins (vRNPs) through the endocytic network and into the nucleus (Kohr et al., 2003). ESCRT machinery recognizes membrane-bound ubiquitinated cargo and forms intraluminal vesicles and targets these vesicles to lysosome, preventing recycling of the cargo back to the plasma membrane (Raiborg and Stenmark, 2009). Thus, ubiquitination may not only influence pathogen receptor cell surface expression and internalization, but may also influence intracellular trafficking. Although the influence of ubiquitin-dependent regulation of adenovirus receptors on adenovirus cell entry is currently undefined, inhibition of the UPS has been shown to differentially influence adenovirus cell entry depending on which primary receptor the virus uses (Shayakhmetov et al., 2003). Thus, we have outlined our current understanding of the potential roles of ubiquitin in the biology of several adenovirus receptors below.
Coxsackievirus and adenovirus receptor (CAR)

The Coxsackievirus and adenovirus receptor is a single pass, type I membrane protein residing in apical junction complexes of polarized epithelial cells (Freimuth et al., 2008). Several recent observations suggest that the localization and cell surface levels of CAR may be regulated by ubiquitination. First, in certain colon cancer cell lines expressing relatively low levels of CAR, treatment of cells with the proteasomal inhibitor, MG132 greatly increased the levels of CAR present on the cell surface as well as the ability of these cells to be transduced with Ad5 vectors (Zhang et al., 2008). Similar observations made for other cell surface receptors have eventually lead to the conclusion that cell surface levels of these receptors are controlled by ubiquitination. Although Zhang, et al. demonstrated that the elevated cell surface CAR levels in MG132 treated cells were not due to increased mRNA levels, but were likely regulated post-translationally, no evidence for CAR ubiquitination was reported.

Further support for the potential regulation of CAR cell surface expression by the UPS comes from the observation that greater cell surface expression of CAR, and hence greater transduction by Ad5 vectors, occurs when extracellular domain of CAR is expressed as a GPI-linked protein compared to expression of CAR containing its transmembrane domain (Wang et al., 2007). Interestingly, the cytoplasmic tail of CAR contains 9 lysines. Although mutational analysis of these lysine residues and its effect on CAR cell surface expression was not performed, these lysine residues may serve as ubiquitination sites. Lack of the cytoplasmic tail could prevent ubiquitin-dependent downregulation of CAR from the cell surface. Alternatively, if CAR is ubiquitinated in
its cytoplasmic tail, ubiquitination may serve as a signal to target CAR for endocytosis and trafficking to lysosomes for degradation.

More specific evidence for a role of ubiquitin in the biology of CAR comes from two findings. First, CAR was found to associate with LNX, a protein known to act as an E3-ubiquitin ligase targeting proteins for degradation (Sollerbrant et al., 2003). However, whether CAR associates with LNX during adenovirus infection, and whether the E3-ubiquitin ligase activity of LNX affect adenovirus cell entry has yet to been examined.

Second, a role for ubiquitination in regulating cell surface levels of CAR comes from a recent report that a recently identified splice-variant of CAR, which is thought to mediate infection of polarized airway epithelium, contains an additional intracellular domain encoded by exon 8 (CARex8) and localizes to apical surfaces of polarized lung epithelia rather than apical junctional complexes (Excoffon et al., 2010). This report also found that cell surface levels of CARex8 were regulated by the PDZ- and WW-domain containing protein MAGI-1b. While both the previously identified version of CAR, CARex7, normally localized to apical junctional complexes, and CARex8 appear to be able to bind or colocalize with MAGI-1b, Excoffon et al. demonstrated that only CARex8 levels are decreased in the presence of MAGI-1b, suggesting that MAGI-1b regulates CARex8, but not CARex7 degradation. More recently, Kolawole et al. demonstrated that two MAGI-1 PDZ domains, PDZ1 and PDZ3, regulate CAR(Ex8) levels in opposing ways (Kolawole et al., 2012). Like full-length MAGI-1, expression of the isolated PDZ3 domain significantly reduces cell surface CAR(Ex8) abundance and
adenovirus infection, whereas the PDZ1 domain is able to rescue CAR(Ex8) and adenovirus infection from MAGI-1-mediated suppression (Kolawole et al., 2012). Although not directly addressed, it would be interesting to determine whether MAGI-1b dependent degradation of CARex8 involves ubiquitination of the CAR cytoplasmic tail. Together, these data suggest that CAR cell surface expression, and perhaps cell surface localization is regulated post-translationally by the UPS. Further studies of UPS regulation of CAR cell surface expression and if CAR is ubiquitinated might provide important insight into adenovirus tropism, particularly as it relates to the use of adenoviral vectors for gene therapy of cardiovascular disease and cancer.

**CD46**

Adenovirus Subgroup B viruses utilize CD46 instead of CAR as their primary receptor. CD46, a membrane complement regulator protein which can bind and inactivate C3b and C4b (Oglesby et al., 1992), is expressed as several alternatively spliced isoforms containing 4 short consensus repeats, 1-3 serine/threonine-rich domains, a transmembrane domain and a cytoplasmic tail (Seya et al., 1999). Only consensus repeats 1 and 2 in the extracellular domain are required for Ad-CD46 interaction (Fleischli et al., 2005; Gaggar et al., 2005; Sakurai et al., 2006). It was hypothesized that the cytoplasmic domain of CD46 could transduce signals. Indeed, CD46 can transduce signals via phosphorylation of the E3-ubiquitin ligase Cbl (Astier et al., 2000). Recent studies indicate Cbl plays a role in ligand-induced ubiquitination, down-regulation, and lysosomal targeting of receptors. Interestingly, measles infection down-regulates CD46, but whether or not CD46 downregulation was dependent on Cbl phosphorylation or Cbl
E3 ubiquitin ligase activity was not explored (Naniche et al., 1993). Further, CD46 downregulation after adenovirus infection has not been reported.

The ubiquitination status of CD46, and if Cbl ubiquitinates CD46, has not been explored. However, Shayakhmetov et al. have demonstrated a role for the UPS during infection by subgroup B human adenovirus serotype 35, which uses CD46 as a receptor (Shayakhmetov et al., 2003). Using a recombinant Ad5 in which the fiber was swapped with the adenovirus type 35 fiber protein in the capsid (Ad5/F35), Shayakhmetov et al. found that treatment with the proteasome inhibitor MG132 reduced infection of HeLa cells by Ad5/F35 compared to untreated cells, whereas Ad5 infectivity was unchanged with MG132 treatment (Shayakhmetov et al., 2003). Further studies are needed to determine the role of ubiquitination in the regulation of CD46-endocytosis and trafficking during adenovirus cell entry.

\( \alpha_v \text{ Integrins} \)

Integrins, present in all nucleated cells, play a role in a number of cellular processes, including cell adhesion, migration, growth, and differentiation. Integrins are heterodimers composed of an alpha and beta subunit. Engagement of \( \alpha_v\beta_1 \), \( \alpha_v\beta_3 \) or \( \alpha_v\beta_5 \) by the penton base of all but subgroup F adenoviruses can trigger clathrin-mediated endocytosis (Nemerow et al., 2009; Smith et al., 2010b). Interestingly, several observations suggest that the \( \alpha_v \) integrin used by adenovirus for endocytosis greatly influences intracellular trafficking of the virus (Wickham et al., 1994). Specifically, use of \( \alpha_v\beta_3 \) targets the virus for lysosomes, resulting in reduced infectivity (Wickham et al., 1994). Interestingly, several observations suggest that ubiquitination of \( \alpha_v\beta_3 \) integrins
may occur upon receptor ligation. First, Tsai et al. demonstrated that Streptococcal pyrogenic exotoxin B (SPE-B) intoxication depends, in part, on binding of SPE-B to αvβ3 via an RGD motif (Tsai et al., 2008). Additionally, the cell surface levels of αvβ3 were decreased after SPE-B binding, and surface levels were restored with treatment of the proteasome inhibitor MG132, suggesting that αvβ3 internalization and, perhaps recycling is regulated by the ubiquitin-proteasome system (Tsai et al., 2008).

Second, Thomas et al. demonstrated that αvβ3 integrin is ubiquitinated in human umbilical vein endothelial cells in response to angiopoietin-2, although whether the alpha or beta chain is ubiquitinated was not described (Thomas et al., 2010). They show that αvβ3 integrin is internalized and ubiquitinated after 10 minutes of angiopoietin-2 ligand treatment (Thomas et al., 2010). No ubiquitination of αvβ3 integrin was detected in the absence of angiopoietin-2 treatment, demonstrating that ubiquitination of αvβ3 integrin requires ligand binding. However, it was not determined if αvβ3 integrin was ubiquitinated at the cell surface, resulting in receptor internalization or if ubiquitination occurred post internalization. Additionally, αvβ3 integrin colocalized with the lysosome marker LAMP1 after 1 hour of angiopoietin-2 treatment, but whether αvβ3 integrin trafficking to lysosomes required ubiquitination was not determined (Thomas et al., 2010).

To date, no studies determining if αvβ5 integrin is ubiquitinated upon ligand binding have been performed. Initial reports demonstrated that αvβ5 can more greatly support Ad5 infection and endosomal escape than αvβ3 integrins (Wickham et al., 1994). Subsequent studies found that reduced expression of β5 integrin leads to greater targeting
of Ad5 to lysosomes (Carey et al., 2007). Although, Carey et al. did not determine which integrin β-subunit was utilized in cells with lower β5 expression. Nonetheless, it would be interesting to determine whether differences in the ubiquitination of αvβ3 and αvβ5 integrin are observed during Ad5 cell entry and whether integrin ubiquitination correlates with lysosomal trafficking of Ad5.

**Adenovirus Activation of Immune Responses**

While required for infection, virus uncoating and membrane rupture also activates a number of immune pathways. Exposure of the Ad double-stranded DNA genome activates TLR9, activating the transcription factor NFκB (Muruve et al., 2008; Barlan et al., 2011a; Barlan et al., 2011b). After NFκB activation, it translocates to the nucleus and initiates the transcription of many proinflammatory cytokines. Appledorn et al. found that when TLR9 signaling is inhibited during Ad infection in mice, there are lower circulating levels of IL-6 and MCP-1 1 hour after i.v. injection, and lower levels of MCP-1, RANTES and IL-12 at 8 hours post injection (Appledorn et al., 2008). Additionally, the amount of TLR9 stimulation during Ad infections influences dendritic cell maturation, which can influence the adaptive immune response (Perreau et al., 2012).

A number of reports indicate that Ad-receptor interactions and endosomal trafficking influence the proinflammatory response induced by Ad cell entry. For example, vaccination with Ads that utilize CD46 as their primary receptor traffic to late endosomes/lysosomes prior to endosomal escape elicit higher levels of cytokines compared to the subgroup C viruses, which utilize CAR as their primary receptor and traffic to early endosomes prior to escape (Miyazawa et al., 2001; Shayakhmetov et al.,
Additionally, replacing the fiber protein on Ad5 so that it utilizes CD46 increases secretion of the IL-1β cytokine (Barlan et al., 2011a). Ads preincubated with serum containing Ad-specific antibodies exhibited increased innate immune responses compared to Ads preincubated with naïve serum, indicated by increased NFκB activation, type 1 interferon production and IL1-β secretion (Zaiss et al., 2009b). The increased innate immune was attributed to the Ad-specific antibodies targeted Ads to phagolysosomes in macrophages compared to Ads preincubated with naïve serum (Zaiss et al., 2009b). Finally, although both αvβ3 and αvβ5 integrins equally promote Ad internalization, interaction with αvβ5 promotes membrane permeabilization and genome delivery, while engagement of αvβ3 targets Ads to lysosomes (Wickham et al., 1994; Carey et al., 2007). Although viruses interacting with αvβ3 exhibited decreased membrane permeabilization, it would be interesting to see how the innate immune response compares to αvβ5 utilization, since membrane permeabilization from lysosomes leads to an increased immune response.

Membrane rupture during Ad entry serves as a danger signal, significantly contributing to the inflammatory response. Membrane rupture by viruses, bacteria and particulates releases activated cathepsins into the cytosol, generating reactive oxygen species due to mitochondrial membrane damage (Dostert et al., 2008; Hornung and Latz, 2010; McGuire et al., 2011; Dupont, 2009 #2). Ad activation of the NLRP3 inflammasome requires generation of reactive oxygen, as viruses that do not uncoat or inhibiting reactive oxygen species do not activate the NLRP3 inflammasome (Muruve et al., 2008; Barlan et al., 2011b; McGuire et al., 2011). Activation of the NLRP3
inflammasome leads to cleavage of pro-IL-1β and pro-IL-18 into their mature forms, which can be secreted from cells (Petrilli et al., 2007). Ad membrane rupture ultimately allows for endosomal escape, and Ad recognition by RIG-I in the cytosol activates IRF3, which generates IFNβ (Minamitani et al., 2011).

Activation of the innate immune response generates an adaptive immune response to Ads, providing protection against subsequent Ad infections. Neutralizing antibodies develop within one week of a primary infection in mice (Zaiss et al., 2009a). These neutralizing antibodies are specific to the Ad fiber, hexon, and penton base capsid proteins, although the majority of the neutralizing antibodies generated are to the hexon (Chirmule et al., 1999; Sumida et al., 2005; Zaiss et al., 2009a). CD8+ cytotoxic T cells are generated against viral gene products and CD4+ Th1 cells are generated against Ad capsid components (Chirmule et al., 1999; Nayak and Herzog, 2010).

**Galectins as Markers for Vacuole Lysis**

Recently, Paz et al. identified the cytosolic protein galectin-3 (gal3) as a marker for bacterially ruptured phagosome membranes (Paz et al., 2010). Gal3, a member of the galectin family, contains a consensus sequence in its carbohydrate binding domain (CRD), which has affinity for β-galactoside containing glycoconjugates (Barondes et al., 1994; Houzelstein et al., 2004; Leffler et al., 2004). The cytosolic gal3 is recruited to disrupted membranes by binding N-linked glycans found on the exterior surface of the plasma membrane when they are exposed from the interior vacuole surface following lysis (Paz et al., 2010). Membrane rupture can be monitored via immunofluorescence microscopy (IFA). Gal3 staining in the absence of membrane rupture appears diffuse,
whereas staining after being recruited to ruptured membranes appears as punctate (Paz et al., 2010). In addition to gal3, galectins 8 and 9 are also recruited to bacterially ruptured phagosome membranes (Thurston et al., 2012). We recently demonstrated that gal3 is a marker for Ad5 membrane rupture (Maier et al., 2012).

**Autophagy**

Autophagy is a host cellular mechanism of sequestering cytoplasmic organelles and molecules. There are three main types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Yang and Klionsky, 2009). During macroautophagy (hereafter referred to as autophagy) a double-membrane vesicle, termed the autophagosome, forms around and sequesters the cytoplasmic contents. Autophagosomes fuse with other membrane-bound compartments, such as endosomes or lysosomes (Yang and Klionsky, 2009). Degradation of cellular proteins and organelles in lysosomes provide free amino acids for new protein synthesis.

Autophagy can also serves as a defense mechanism against intracellular pathogens in a number of ways. Sequestration of pathogens in autophagosomes and subsequent fusion with lysosomes can degrade the pathogen, limiting their replication and spread (Levine and Deretic, 2007). Additionally, degradation of pathogens in lysosomes increases pathogen peptide presentation on MHC-II for presentation to CD4+ T cells (Levine and Deretic, 2007; Nedjic et al., 2009; Gannage and Munz, 2010; Munz, 2010). Third, autophagosomes can sequester viral genomes in the cytosol and fuse with endosomes where the genome is recognized by TLRs, activating the production of Type I interferons (IFNs) (Levine and Deretic, 2007).
To date, most research focused on whether a particular pathogen infection regulates autophagy. The cellular status of the autophagosome marker, LC3, monitored by IFA or Western Blotting is the classical method of determining if autophagy is upregulated in response to a particular stimulus (Tanida et al., 2004; Mizushima and Yoshimori, 2007). LC3-I is the form located throughout the cytosol of the cell. In the absence of autophagy, IFA of LC3 appears diffuse. LC3 is cleaved and lipidated when autophagy is activated and LC3 is recruited to autophagosome membranes (Tanida et al., 2004; Mizushima and Yoshimori, 2007). This lipidated form is known as LC3-II, and appears as punctate staining by IFA. The lipidation of LC3 results in an electrophoretic shift, which can be monitored by Western Blot (Mizushima and Yoshimori, 2007). In many cell types there are basal levels of autophagy always occurring and increases in autophagosome formation are measured by an increase in the number of LC3 puncta per cell or amounts of LC3-II present (Tanida et al., 2004; Mizushima and Yoshimori, 2007).

Many intracellular pathogens contain proteins that inhibit autophagy during infection (Munz, 2007; Orvedahl and Levine, 2008). For example, the HSV-1 ICP34.5 and KSHV viral Bcl-2 proteins bind to Beclin-1 (Pattingre et al., 2005; Orvedahl et al., 2007). When these viral proteins bind to Beclin-1 during infection, Beclin-1 cannot interact with Vps34, required for LC3 recruitment and autophagosome formation (Furuya et al., 2005; Funderburk et al., 2010; Yue and Zhong, 2010). Thus, these viruses have evolved a mechanism to prevent autophagy induction that would limit their infection and pathogenesis. For some viruses, however, replication requires autophagy, as these viruses replicate on the double-membrane vesicles (reviewed in (Shi and Luo, 2012)).
Human immunodeficiency virus (HIV) overactivates autophagy to allow for virus infection (Blanchet et al., 2010).

Little is known about how pathogens, their proteins, and their genomes are specifically targeted to autophagosomes. Two proteins, p62/SQSTM1 and neighbor of BRCA1 (NBR1), have been identified to bind polyubiquitinated proteins and target them to autophagosomes by binding to LC3 (Kirkin et al., 2009; Lamark et al., 2009). Several reports have demonstrated that membrane fragments positive for galectin staining are targeted for degradation via autophagy (Dupont et al., 2009; Thurston et al., 2012). Dupont et al. showed that the autophagosome marker, LC3, is recruited to gal3 positive membrane fragments during Shigella infection (Dupont et al., 2009). Thurston et al. demonstrated that galectins 3, 8, and 9 are recruited to Salmonella enterica (Thurston et al., 2012). Additionally, nuclear dot protein 52kDa (NDP52), which binds LC3, binds galectin 8 (Thurston et al., 2012). Only knockdown of galectin-8 significantly decreased LC3 recruitment to Salmonella and increased bacterial replication (Thurston et al., 2012).

**Activation of Autophagy During Adenovirus Infection**

Autophagy activation during adenovirus infection has been minimally studied. Autophagy activation during adenovirus infection has only been demonstrated at late times during infection (after 24 hours) (Jiang et al., 2008; Jiang et al., 2011; Piya et al., 2011; Rodriguez-Rocha et al., 2011). LC3-II was not detected at 12 hours post infection, but earlier time points during adenovirus infection have not been reported (Rodriguez-Rocha et al., 2011). LC3-II levels diminish after the autophagy stimulus has stopped, so it is feasible to hypothesize that one stimulus activated autophagy early during infection
and then a second, separate stimuli can activate autophagy at late time points during adenovirus infection (Mizushima and Yoshimori, 2007).

Overall, there is mounting evidence that ubiquitination influences, or could influence Ad cell entry. While a link between the autophagy pathway and ubiquitination has been well established, the autophagy status and if autophagy influences Ad cell entry has not been tested. In addition to provide further detail of Ad membrane rupture and endosome escape, this dissertation provides evidence that the pVI-PPxY motif evades autophagy during Ad cell entry.
CHAPTER II
MATERIALS AND EXPERIMENTAL METHODS

Cell lines and viruses. Tissue culture reagents were obtained from Mediatech and HyClone. U2OS, HeLa, THP-1 and A549 cells were purchased from ATCC. 293β5 cells were a kind gift from Glen Nemerow (Smith et al., 2010a). U2OS, HeLa, 293β5 and A549 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 1 mg/ml streptomycin, 0.25 μg/ml amphotericin B, non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer and 2 mM glutamine. THP-1 cells were maintained in RPMI1640 with the same supplements as DMEM. The Ad5-WTgfp and Ad5-M1gfp viruses were previously described (Wodrich et al., 2010). All viruses were propagated in 293β5 cells and purified from cellular lysates by double banding in cesium chloride gradients and dyalized in 40 mM Tris, 150 mM NaCl, 10% glycerol, and 1 mM MgCl₂ (pH 8.2) (Wiethoff et al., 2005). For these studies, the ts1 virus (Weber, 1976) was propagated at the nonpermissive temperature of 39.5 °C (Cotten and Weber, 1995; Greber et al., 1996; Wiethoff et al., 2005). Viral concentrations were determined by Bradford assay (Bio-Rad Laboratories, Inc.) and aliquots were flash frozen in liquid nitrogen and stored at –80°C.

Reagents, siRNAs and Antibodies: Phorbol-12-myristate-13-acetate (PMA), Nocodazole (NOC) and 3-Methyladenine (3-MA) were purchased from Sigma-Aldrich.
Control and Atg5 siRNAs were purchased from Cell Signaling. Galectin-8 siRNA was generated by Dharmacon to the following target site sequence: 5’-GGCCUUUCAUUUCAAUCCU-3’ (Mobergslien and Sioud, 2011). The following primary antibodies were used: rabbit LC3, rabbit p62, rabbit NDP52, mouse β-actin (Sigma-Aldrich), mouse galectin-3 (BD Biotechnology), rabbit Atg5 (Cell Signaling), mouse (EBioscience) and rabbit (Thermo Scientific) CD107a (LAMP-1), mouse (R&D Systems) and rabbit (Epitomics) galectin-8, rabbit and mouse EEA1 (EPIT MICS). pVI was detected with rabbit polyclonal antiserum raised against purified preprotein VI (Maier et al., 2010).

**Infectivity Assays:** Cells were plated in 24 well plates to yield 0.5-1x10^5 cells/well the day of infection. Cells were collected 18-24 hours after infection, pelleted, and resuspended in buffer (PBS with 1% FBS + 0.1% sodium azide) and GFP+ cells were counted via flow cytometry (Accuri or Canto). For 3-MA treated cells, cells were pretreated with 5mM 3-MA for 30 minutes, infected in the presence of 5mM 3-MA for 2 hours. Media was removed and cells were washed and replaced with media containing 5mM 3-MA for 2 hours. Cells were washed again and replaced with DMEM overnight. For nocodazole treated cells, cells were pretreated for with 30μM for 2 hours and infected in the presence of 30μM overnight.

**siRNA Knockdown:** HeLa cells were plated in 24 well plates and transfected with each siRNA using Lipofectamine 2000 (Invitrogen) or TransIT-siQUEST® reagent (Mirus). Cells were transfected with 20 pmol (Day 1) and with 30 pmol (Day 2) with each siRNA per well. Cells were infected seventy-two hours after the first transfection.
Immunofluorescence Microscopy: A total of 2.5-5×10^4 cells were plated on glass coverslips. The next day the cells were infected with 1×10^4 vp/cell on ice for 1 hour after which the cells were shifted at 37°C to allow for virus internalization. At different times post virus internalization the cells were washed with PBS and fixed with 3.7% paraformaldehyde (Electron Microscopy Sciences) in 0.159M PIPES buffer (Sigma) for 10 min. Cells were then blocked for 1 hr in PBS with 10% FBS and 0.5% saponin (Sigma). Staining with specific mono- or polyclonal antibodies was done in 10% FBS with 0.5% saponin for 1 hr. Secondary Alexa Fluor 568-conjugated (Invitrogen) and DyLight 649-conjugated (Jackson) antibodies were used. Viruses were prelabeled with Dylight 488 NHS-Ester Fluorophores (Thermo Scientific), according to the manufacturer's protocol prior to use. DAPI was used to counterstain nuclei before coverslips were mounted on glass slides with ProLong Gold (Invitrogen) or Flouro-Gel (Electron Microscopy Sciences). Z-stack images were acquired using identical parameters for each timepoint with a DeltaVision microscope (Applied Precision) using a CoolSnap HQ digital camera (Photometrics) with a 1.4-numerical aperture (NA) 100× objective lens, and deconvolved with SoftWorx deconvolution software (Applied Precision). Images were assembled using IMARIS and ImageJ softwares. Virus particles, LC3 puncta and galectin puncta three-dimensional surfaces were acquired using IMARIS software. Colocalization was determined by the fluorescence intensity of the protein of interest at each three-dimensional surface compared to the fluorescence intensity of the secondary only control.

Immunoblotting: Cells were lysed in a solution containing 25mM Tris, 25mM NaCl,
0.1mM EDTA, 1% tritionX-100, 0.5% NaDeoxycholate, 0.1% β-mercaptoethanol and 1mM PMSF, ran on 15% SDS gels and transferred to PVDF membranes (Millipore). Membranes were probed either overnight at 4°C or at room temperature for 1-2 hours in TBST 0.1% with 5% BSA or PBST 0.05% with 5% milk. Mouse and rabbit HRP-conjugated secondary antibodies were used (Thermo Scientific). Membranes were developed with Pierce Super Signal or Dura substrates on GE or Denville film.

**ELIspot and ELISA Assays:** Human PBMCs were obtained from healthy volunteers. 0.5-10x10^6 monocyte-derived dendritic cells were transduced with virus or media alone for 2 hours. Cells were incubated with CD4+ T-cell clones that had been cultured for 4 weeks to recognize a conserved Ad5 hexon epitope at a 3:1 ratio overnight. Supernatants were collected and ELISA was performed per manufacturer’s protocol (eBioscience).

For ELISpot assays, B6 mice were infected with 10^{10} viral particles or PBS control intramuscularly. Mice were sacrificed and whole splenocytes were collected by filtering the spleen through a 100 μm filter. Cells were pelleted at 400 x g, red blood cell lysis buffer (Sigma) was added for 2 minutes and resuspended in PBS and splenocytes were pelleted at 400 x g. Splenocytes were plated into ELISpot plates (Millipore) and stimulated with Ad5-luc capsids or GFP purified from *E. coli* for 48 hours. ELISpot was performed per manufacturer’s instructions (Millipore). Spots were read on Cellular Technology Ltd reader with Immunospot software. Data are reported as the number of spot forming colonies (SFC)/10^5 PBMCs. All animal experiments were approved and performed in compliance with institutional policies.

**Statistical Analysis:** Statistical significance (p < 0.05) was assessed using the Student's *t*
test whenever two groups were compared. Data are presented as mean ± SEM.

Calculations were performed with Microsoft Excel (Microsoft, Inc.) or GraphPad Prism software (GraphPad Software, Inc.).
CHAPTER III

RESULTS

The kinetics and subcellular localization of Ad5 membrane rupture and endosomal escape

Ad5 membrane rupture occurs near the cell surface

Recent reports suggest that Ad5 uncoating occurs early during infection, near the cell surface by determining when the interior capsid protein pVI was accessible to antibody staining (Wodrich et al., 2010; Burckhardt et al., 2011). However, they did not determine when Ad had ruptured membranes. We have found that the cytosolic protein gal3 can be used as a marker for Ad membrane rupture (Maier et al., 2012). Gal3 labels disrupted vacuolar membranes by binding cell surface N-linked glycans when they are exposed following vacuole lysis, which can be visualized using IFA as punctate structures (Paz et al., 2010). Using synchronous infections, we visualized Ad-induced gal3 punctate structures were within 20 minutes of shifting the cells to 37°C to allow for endocytosis, which was after we observed pVI exposure (Maier et al., 2012). When we imaged Ad5-induced gal3 puncta in fixed cells, we often observe Ad5 in gal3 punctate structures near the cell surface at early times post infection (Figure 2A). Colocalization of gal3 and pVI with labeled virions was observed at 30 minutes post-warming at, or very near, the cell surface (Figure 2A).

To determine the kinetics of Ad5 membrane rupture in real time, the
Figure 2. Ad5 membrane rupture occurring near the cell surface. A. HeLa cells were infected with fluorescently labeled Ad5 and fixed at 0 and 30 minutes post infection, stained for gal3 (red) and pVI (blue). Nuclei were stained with DAPI (grey). Bar represents 5μm. B. U2OS cells stably expressing mCherry-gal3 were infected with Alexa488-Ad5 and images were collected 15 min post-infection and collected at 5Hz. Right panels are time-lapse still images of viral particle (white box, left panel) acquiring mCherry-gal3.
osteosarcoma cell line, U2OS, stably expressing mCherry-gal3 was infected with Alexa488-labeled Ad5 and cells were subjected to live cell imaging. As seen by immunostaining for endogenous gal3, in uninfected cells mCherry-gal3 appeared diffuse throughout the cytoplasm of cells (data not shown). Upon Ad5 infection, punctate gal3 fluorescence was observed. For technical reasons we used asynchronous infections performed at 37°C without pre-binding of virus. We observed that recruitment of maximal mCherry-gal3 to Ad5 ruptured membranes was rapid, typically taking less than 1s from the first observation of colocalization to maximal colocalization of all pixels attributed to a single virion (Figure 2B). This also occurred near the cell surface. Our studies correlate with previous reports that exposure of pVI occurs early in infection near the cellular surface.

*Exposure of pVI occurs prior to trafficking to EEA-1 + early endosomes*

Several lines of evidence suggest that Ad5 traffics through early endosomes prior to endosomal escape (Gastaldelli *et al.*, 2008; Moyer *et al.*, 2011). To examine in more detail the compartmentalization of pVI release, we determined Ad5 colocalization with pVI and the early endosomal marker, EEA-1. To examine the compartmentalization of Ad5 membrane rupture, we determined Ad5 colocalization with gal3 and EEA1 or the late endosome/lysosome marker LAMP1. Fluorescently labeled Ad5 was added onto HeLa cells on ice to allow the virus to bind, but not endocytose. After 1 hour of binding, cells were washed with ice cold media to wash away the unbound virus, and cells were shifted to 37°C by adding pre-warmed media and placed in the incubator (Time 0) to allow for Ad5 endocytosis. Cells were fixed at various time points post infection and
stained with antibodies to pVI or gal3 and EEA1 or LAMP1.

We observed colocalization of Ad5 with pVI beginning at 10 minutes post infection, with maximal colocalization occurring at 20 minutes post infection (Figure 3A). While 52% of Ad5 colocalized with pVI 20 minutes post infection, less than 25% of incoming Ad5 colocalized with both pVI and EEA-1. This suggests that approximately half of viral uncoating occurs prior to or outside of early endosomes.

To determine whether gal3 recruitment to Ad5 occurs in a subset of endosomal compartments, analogous experiments were performed by immunostaining for gal3 and EEA-1 or LAMP-1. Maximum colocalization of Ad5 with gal3 occurred 20-40 minutes post infection (Figure 3B). Less than half of the Ad5 that colocalized with gal3 also colocalized with EEA-1 (Figure 3B). We propose that the majority of Ad5 virions do not rupture membranes in early endosomal compartments. It is even less likely that Ad5 ruptures membranes in late endosomes/lysosomes as less than 2% of Ad5 colocalizing with gal3 also colocalized with the late endosome/lysosome marker LAMP1 (Figure 3C). Taken together these data suggest that pVI exposure occurs prior to virus trafficking to early endosomes or in endosomal compartments not containing the common early and late endosomal markers, EEA-1 and LAMP-1.

Characterization of Ad5 endosome escape

Currently, there is limited data to describe Ad endosome escape. Longberg-Holm et al. used $^{32}$P-labeled Ad serotype 2 (Ad2, a subgroup C member) and cell fractionation assays to determine when the virus was no longer associated with membranes, and estimated that the half life of Ad2 in endosomes is approximately 15 minutes (Lonberg-
Figure 3. Ad5 acquires pVI and gal3 prior to early endosomes. Fluorescently labeled Ad5 (green) was bound to HeLa cells for 1 hr at 4°C after which cells were washed and shifted to 37°C. Cells were fixed and stained for gal3 (red) and pVI (A) EEA-1 (B) or LAMP-1 (C). Merged images (M). The percent of Ad5 colocalization was determined. Bar represents 5 μm.
Holm, 1969). Moyer et al. correlated Ad5 endosome escape with colocalization of the early endosome marker EEA1, attributing lack of colocalization with EEA1 meant Ad5 had already escaped the endosome (Moyer et al., 2011). Electron microscopic studies of Ad infected cells have been able to capture the virus at the cell surface, in an intact endosome or in the cytoplasm, but have been unable to adequately describe Ad crossing a disrupted endosomal membrane (Nakano et al., 2000; Gastaldelli et al., 2008; Imelli et al., 2009). Using gal3 as a marker depicting ruptured endosomes, we asked if Ad5 ruptured membranes and subsequently escaped these endosomes, or if they could traffic within these ruptured membranes.

During live cell imaging of our U2OS cells that stably express mCherry-gal3, colocalization of gal3 with Ad5 had a surprisingly long half-life, as many viruses remained colocalized with gal3 puncta for the duration of the movie (~320s). We also observed Ad5 movement within the cell while colocalized with gal3 (not shown). This suggests that membrane rupture is temporally and spatially distinct from endosomal egress. It also suggests that pVI membrane rupture is not sufficient for the virus to escape from endosomes.

Using live cell imaging of asynchronously infected cells, 30 min after addition of the virus, we also characterized endosomal egress events. In Figure 4, Ad5 is shown initially in gal3+ endosomes which are predominantly perinuclear and relatively immobile. Whether these viruses traffic to this perinuclear location in gal3+ endosomes, and whether the endosomal membranes remain disrupted, or resealed is unknown. These Ad5 particles can escape these gal3+ punctate structures and upon egress, Ad5 undergoes
Figure 4. Time-resolved imaging of Alexa488-Ad5 endosomal escape in U2OS cells expressing mCherry-gal3. Images were collected 30 min after infection at a rate of 5 Hz. Particle tracking performed with MTrackJ.
rapid directional movement, as indicated by traces (Figure 4). This rapid, directional movement is in agreement with previous observations of microtubule-dependent movement of the virus in the cytoplasm (Greber et al., 1993; Suomalainen et al., 1999; Engelke et al., 2011; Strunze et al., 2011).

**Microtubule contribution during Ad5 cell entry**

*Microtubules contribute to Ad5 endosome escape*

Several reports demonstrate that disruption of microtubules, either by pharmacological agents or siRNA knockdown of microtubule motor components, decreases Ad infection (Suomalainen et al., 1999; Leopold et al., 2000; Mabit et al., 2002; Engelke et al., 2011; Strunze et al., 2011). When Ad engages microtubules is not completely understood. Leopold et al. reported translocating Ads in a neutral pH environment, concluding that Ad engagement of microtubules was not in acidified endosome and, instead, occurred after endosome escape (Leopold et al., 2000). However, accumulating data suggest that subgroup C viruses (which includes Ad5) do not need low pH exposure for uncoating (Rodriguez and Everitt, 1996; Nakano et al., 2000; Gastaldelli et al., 2008; Wodrich et al., 2010; Burckhardt et al., 2011; Maier et al., 2012). Additionally, Leopold et al. did not directly determine if these translocating Ads were associated with membranes, or free virus in the cytosol. Since we observed that Ad5 undergoes rapid, directional movement upon egress from gal3+ puncta (Figure 4), we determined if microtubules are needed for Ad5 endosomal escape.

HeLa cells were pretreated with 30μM NOC and fluorescently-labeled Ad5-WT was added to pre-cooled cells for 1 hour on ice to allow Ad5 to bind, but not endocytose.
Unbound virus was washed away and cells were shifted to 37°C (Time 0). Cells were fixed at various times post infection and stained with antibodies to gal3 and pVI. Using immunofluorescence microscopy (IFA) and image analysis software (IMARIS®), we observed that NOC treatment did not affect Ad5 endocytosis or membrane rupture, as the number of gal3 puncta in NOC-treated cells was not significantly different compared to untreated cells at early times post infection (Figure 5A and 5B). However, Ad5 colocalization with gal3 increased significantly in cells treated with NOC at 1 and 2 hours post temperature shift, suggesting that microtubules are needed for efficient endosomal escape (Figure 5C). We have previously shown that colocalization of Ad5 with gal3 puncta decreases over the course of infection, while pVI remains colocalized with gal3 puncta (Maier et al., 2012). We also observed increased Ad5 colocalization with pVI in cells treated with NOC (Figure 5D). These results demonstrate that microtubules are needed for efficient Ad5 endosomal escape.

**Ad5 microtubule engagement is mediated via the pVI-PPxY motif**

*The pVI-PPxY motif increases Ad5 nuclear accumulation*

Recently, a mutant Ad5 was generated that exhibits decreased specific infectivity, but no defect in membrane rupture (Wodrich et al., 2010) and Figure 6A-C). This mutant virus contains a mutation in the conserved pVI-PPxY motif (Ad5-M1). Overexpression studies with RFP-tagged pVI show that the pVI-PPxY motif is needed for pVI colocalization with tubulin, and that Ad5-M1 virions have decreased accumulation at the microtubule organizing center (MTOC) (Wodrich et al., 2010). Since Ad5 accumulates at the perinuclear region, which is dependent on microtubules (Mabit et al., 2002), we
Figure 5: Microtubules aid in Ad5 endosome escape. HeLa cells were infected with Ad5-WT (purple) in the presences or absence of NOC, fixed and stained for gal3 (green) and pVI. A. Representative images of 30min and 2hr time points. Bar represents 8μm. Arrows indicate colocalization. Quantification of the number of gal3 puncta per cell (B.) and Ad5 colocalization with gal3 (C.) and pVI (D.)
Figure 6: Ad5-M1 exhibits decreased specific infectivity, but not decreased membrane rupture. HeLa cells (A) or A549 cells (B) were infected with varying concentrations of physical particles per cell with Ad5-WTgfp or Ad5-M1gfp for 18-24 hours. The percent GFP+ cells for each condition were determined by FACS. C. Increasing numbers of virus particles were added to HeLa cells in the presence of the ribosomal toxin saporin. Cell viability was determined by measuring the absorbance at 550nm after the addition of MTT. Values are normalized to no virus added.
Figure 7: The pVI-PPxY motif is needed for Ad5 microtubule trafficking to the nucleus. HeLa cells were infected with Ad5-WT or Ad5-M1 (green) in the presence or absence of NOC. A. Representative images of 0hr and 2hr timepoints. Bar represents 5μm. Gal3 is in red. B. Quantification of Ad5 at the perinuclear region, defined as 1.5μm around the nucleus.
hypothesized that the pVI-PPxY motif aids in Ad5 nuclear accumulation.

We quantified Ad5 accumulation at the perinuclear region on fixed cell images of synchronous infections by calculating the percent of Ad5 virions within a 1.5\(\mu\)m radius of the nucleus in the presence of absence of NOC using ImageJ software. NOC treatment decreased Ad5-WT accumulation at the perinuclear region (Figure 7A and 7B), as had previously been reported (Mabit et al., 2002). We saw a similar defect in perinuclear accumulation with Ad5-M1 and NOC treatment did not further decrease Ad5-M1 accumulation (Figure 7A and 7B), suggesting that the pVI-PPxY motif is needed for Ad5 trafficking to the nucleus. Since microtubules are involved in Ad perinuclear accumulation, we conclude that the pVI-PPxY motif is defective for either Ad5 recruitment of microtubules and/or Ad5 engagement of microtubules.

*The pVI-PPxY motif increases Ad5 endosome escape*

We observed that depolymerizing microtubules using NOC increased Ad5-WT colocalization with gal3 and decreased Ad5-WT accumulation at the nucleus, suggesting that microtubules are needed for efficient Ad5 endosomal escape and perinuclear accumulation. Since we observed decreased Ad5-M1 perinuclear accumulation, and that accumulation was not further increased with the addition of NOC, we asked if Ad5-M1 was also deficient in endosome escape. Using the same IFA protocol, we infected HeLa cells with Ad5-WT or Ad5-M1 and determined colocalization with gal3 and pVI. If the pVI-PPxY motif is needed for Ad5 endosomal escape, then we would see increased colocalization of Ad5-M1 with gal3 and pVI compared to Ad5-WT. There was no significant difference observed in the number of gal3 puncta per cell between Ad5-WT
Figure 8: Ad5 endosome escape is pVI-PPxY dependent. Fluorescent-labeled Ad5-WT or Ad5-M1 (green) was added to cells on ice for 1 hour. Unbound virus was washed away and cells were shifted to 37°C to allow for endocytosis. Cells were fixed and stained for gal3 (red). A. Micrographs of Ad-WT and Ad-M1 infected HeLa cells at 30 minutes post temperature shift. Bar represents 5μm. Quantification of the number of gal3 puncta per cell for HeLa (B.) and U2OS (D.) cells and the percent colocalization of Ad5 with gal3 in HeLa (C.) and U2OS (E.) cells. **p<0.005
and Ad5-M1 infected cells (Figure 8A and 8B), supporting previous studies that mutation of the pVI-PPxY motif does not affect membrane rupture (Wodrich et al., 2010 and Figure 6C). However, the percent of Ad5-M1 that colocalized with gal3 or pVI was significantly increased compared to Ad5-WT (Figures 8C and data not shown), suggesting that the pVI-PPxY motif is required for efficient Ad5 endosomal escape. We also tested gal3 puncta formation and colocalization between Ad5-WT and Ad5-M1 in U2OS cells and found similar results (Figure 8D and 8E), demonstrating that the Ad5-M1 defect in endosome escape is not limited to HeLa cells.

Since Ad5-M1 showed increased colocalization with gal3 and pVI similar to Ad5-WT with NOC treatment, we asked if the microtubule engagement needed for Ad5 endosome escape was mediated via the pVI-PPxY motif. To address this question, we infected HeLa cells with Ad5-M1 in the presence and absence of NOC. If microtubule engagement for endosomal escape is mediated via the pVI-PPxY motif, then we would not observe a difference in Ad5-M1 colocalization with gal3 and pVI in the presence of NOC. We again saw no difference in gal3 puncta formation in NOC-treated cells (Figure 9A and 9B). In contrast to the effect we saw on NOC with Ad5-WT gal3 colocalization, we observed no difference with Ad5-M1 colocalization with gal3 or pVI in NOC-treated cells (Figure 9C and 9D). However, when we looked at Ad5-M1 infectivity as measured by the percent of green fluorescent protein (GFP) by flow cytometry, we saw a significant decrease in Ad5-M1 infectivity in cell treated with NOC (Figure 10). The decrease in Ad5-M1 infectivity in cells treated with NOC compared to DMSO control-treated cells was not as severe compared to the decrease of Ad5-WT in the presence of
Figure 9: The pVI-PPxY motif mediates microtubule-mediated endosome escape. HeLa cells were infected with Ad5-M1 (purple) in the presence or absence of NOC. A. Representative images of 30 min and 2hr time points. Bar represents 5μm. Quantification of the number of gal3 puncta per cell (B.) and Ad5-M1 colocalization with gal3 (C.) and pVI (D.)
Figure 10: The effect of NOC on Ad5 infectivity. HeLa cells were treated with 30μm NOC or DMSO for 2 hours prior to infection. Cells were infected in the presence of 30μm NOC or DMSO. The percent of GFP+ cells was determined by FACS. **p<0.001
NOC (Figure 10), suggesting microtubules contribute to Ad5 infectivity independent of endosome escape. From these data, we conclude that the Ad5-pVI-PPxY motif mediates microtubule-mediated endosomal escape and nuclear trafficking.

**Evasion of Autophagy During Ad5 Cell Entry**

*Ad5 activates autophagy during cell entry*

Several reports have demonstrated a link between galectin-decorated bacterially-ruptured membranes and the autophagy cellular degradation pathway (Figure 11). First, gal3 positive membrane fragments contain poly-ubiquitinated proteins, p62/SQSTM1 and the autophagosome marker LC3 (Dupont et al., 2009). p62/SQSTM1 binds to ubiquitin and to LC3 (Pankiv et al., 2007) and in *Shigella* infected Atg4B mutant cells (which are deficient for autophagosome maturation) polyubiquitinated proteins and p62 accumulated on gal3+ membranes (Dupont et al., 2009). Dupont et al. concluded that the gal3+ ruptured membranes were targeted for degradation by autophagy.

Another study by Thurston et al. screened the entire galectin family for recruitment to bacterially-ruptured phagosome membranes (Thurston et al., 2012). They found that in addition to gal3, galectins 8 and 9 were also recruited. They also observed that in addition to p62, a second autophagy receptor, NDP52, which also binds to ubiquitin and to LC3 is recruited to bacterially-ruptured phagosome membranes (Thurston et al., 2012). However, only when gal8 or NDP52 were knocked down by siRNA did they see an increase in bacterial replication (Thurston et al., 2012). Thurston et al. also found that NDP52 can also bind to gal8 (Thurston et al., 2012). Additionally, the gal8 restriction of intracellular bacterial replication was attributed to autophagy, as
Figure 11: Model for degradation of membrane fragments. Galectins binds to ruptured membranes. Ubiquitinated proteins on ruptured membranes are bound by the autophagy cargo adaptors p62 or NDP52. NDP52 can directly bind galectin 8 (not shown) targeting ruptured membranes for autophagy. Autophagosomes fuse with lysosomes for content degradation and presentation by MHC-II molecules.
knockdown of gal8 reduced NDP52 and LC3 colocalization with *Salmonella enterica* (Thurston *et al.*, 2012). They concluded that gal8 recruitment to membrane rupture recruits NDP52, which then recruits LC3 to the damaged membrane and targets them for degradation via autophagy, decreasing bacterial replication (Thurston *et al.*, 2012).

Interactions between the autophagy pathway and viruses during infection have been well documented (Munz, 2007; Lee and Iwasaki, 2008; Orvedahl and Levine, 2008; Deretic and Levine, 2009; Kirkegaard, 2009; Cavignac and Esclatine, 2010; Deretic, 2010; Orvedahl *et al.*, 2010; McFarlane *et al.*, 2011). For example, foot-and-mouth disease virus induces autophagosome formation during entry (Berryman *et al.*, 2012). Autophagy induction during Ad infection has only been demonstrated at later times of infection, after Ad replication has occurred (Jiang *et al.*, 2008; Jiang *et al.*, 2011; Rodriguez-Rocha *et al.*, 2011). The earliest documented time point for autophagy induction during Ad5 infection is 12 hours (Rodriguez-Rocha *et al.*, 2011). While the report claimed that autophagy is not activated until 24 hours post infection, this report used a LC3-luciferase cleavage assay to determine autophagy activation, and whether or not luciferase levels during Ad5 infection are above uninfected cannot be determined (Rodriguez-Rocha *et al.*, 2011). Studies determining autophagy during Ad infection by LC3-II formation by Western Blot have only reported the status of LC3-II no earlier than 24 hours post infection (Jiang *et al.*, 2008; Jiang *et al.*, 2011; Rodriguez-Rocha *et al.*, 2011). When LC3 is recruited to autophagosomes it becomes lipidated, causing an electrophoretic shift, known as LC3-II. However, since LC3-II is turned over by autophagy, it is possible that autophagy is activated earlier during infection independent
of replication, and that LC3-II has been degraded by the 24 hour time point (Mizushima and Yoshimori, 2007). Therefore, we first asked if Ad5 infection induces autophagosome formation.

Again, using synchronized infections, we found that Ad5-WT and Ad5-M1 both induced autophagosome formation, indicated by the presence of endogenous LC3 punctate staining in infected cells within 30 minutes post temperature shift (Figure 12A). Using Imaris® to define LC3 puncta, we found that Ad5-WT and Ad5-M1 infected cells contain similar numbers of LC3 puncta (Figure 12B). We also confirmed autophagy induction during Ad5 infection by Western Blot at 30 minutes post infection (Figure 12C). Ad5-WT and Ad5-M1 induced similar amounts of LC3-II above uninfected background. We conclude that Ad cell entry activates autophagy.

*Mutation of the pVI-PPxY motif targets Ad5 for autophagy*

Ad5-WT and Ad5-M1 infection induced similar numbers of LC3 puncta per cell and similar LC3-II formation, suggesting that the pVI-PPxY motif is not responsible for inhibiting autophagy activation during Ad5 infection. Autophagy can be activated during pathogen infection, but the pathogen may not be affected if it can avoid being degraded by autophagosomes. We asked if the Ad5-WT, which contains the pVI-PPxY motif, avoids being sequestered into autophagosomes. To do this we calculated colocalization of virus and LC3 using IMARIS®. Indeed, the percentage of Ad5-M1 colocalized with LC3 was significantly higher than Ad5-WT at 30 minutes post temperature shift (Figure 13A). Additionally, the percentage of LC3 puncta that contained Ad5-M1 was significantly higher at all time points compared to Ad5-WT (Figure 13B). We also found
Figure 12: Ad5 cell entry induces autophagosome formation. HeLa cells were infected with Ad5-WT or Ad5-M1 (red). Cells were either lysed or fixed and stained for LC3 (green). A. Representative images from 30 min post infection. Circled area shows Ad5-M1 colocalization with LC3 (Quantified in Figure 13). B. Quantification of the number of LC3 puncta per cell. C. Western blot of whole cell lysates probed for LC3.
Figure 13: Ad5 colocalization with LC3 and p62. HeLa cells were infected with Ad5-WT or Ad5-M1 (green). Cells were fixed and stained for LC3 or p62 (red and Figure 12). 

A. Ad5 colocalization with LC3. 
B. LC3 puncta from Figure 12B colocalized with Ad5. 
C. Representative images of p62 staining at 30 min post infection. Bar is 5 μm. 
D. Colocalization of Ad5 with p62. *p<0.01 **p<0.001
that Ad5-M1 colocalized with the LC3-binding adaptor p62, which is found at gal3+ membranes, more than Ad5-WT (Figure 13C and 13D) (Dupont et al., 2009; Thurston et al., 2012). These data indicate that the presence of the pVI-PPxY motif prevents Ad5 sequestration into autophagosomes.

One fate of autophagosomes is fusion with lysosomes; however, some pathogens can prevent autophagosome fusion with lysosomes to prevent their degradation (Birmingham et al., 2008a; Birmingham et al., 2008b). We therefore asked if the Ad5-M1 fate was trafficking to lysosomes. To address this question, we analyzed colocalization of both Ad5-WT and Ad5-M1 with the lysosome marker LAMP1 using IMARIS®. Ad5-M1 colocalized with LAMP1 significantly at higher frequencies than Ad5-WT at later times post infection (Figure 14), with up to 80% of Ad5-M1 colocalizing with LAMP1 at 4 hours post infection. From these data we conclude that Ad5 entry activates autophagy, and that the pVI-PPxY motif is needed to avoid sequestration into autophagosomes and prevent trafficking to lysosomes.

Our data suggest that although both viruses induce autophagosome formation during Ad entry, only Ad5-M1 is being sequestered in autophagosomes and delivered to lysosomes. If autophagy, and subsequent lysosomal trafficking, is responsible for the Ad5-M1 specific infectivity defect, then inhibiting autophagy should restore Ad5-M1 infectivity. To do this we used siRNA to knock down Atg5, which is required for autophagosome formation (Mizushima et al., 2001; Suzuki et al., 2001; Mizushima et al., 2003). There was no difference between Ad5-WT and Ad5-M1 infectivity in Atg5 siRNA knockdown cells (Figure 15A). We also saw an increase in Ad5-M1 infectivity
Figure 14: Ad5 colocalization with LAMP1. HeLa cells were infected with Ad5-WT or Ad5-M1. Cells were fixed and stained for LAMP1. Ad5 colocalization with LAMP1 was determined using IMARIS® software compared to the secondary only antibody control. *p<0.01, **p<0.001
Figure 15: Inhibition of autophagy restores Ad5-M1 infectivity. HeLa cells were transfected twice with control or Atg5 siRNA. Cells were infected with 30 particles per cell of Ad5-WT or Ad5-M1 72 hours after the first transfection. A. The percent of GFP+ cells were determined 18 hours post infection by FACS. * p<0.05 B. HeLa cells were pretreated with 5mM 3-MA for 2 hours and then were infected with increasing concentrations of Ad5-WT or Ad5-M1 in the presence of 3-MA. The percent of GFP+ cells were determined 18 hours post infection by FACS. C. Representative Images of Ad5-M1 (blue) in control and Atg5 (green) siRNA transfected cells at 4hrs post infection with LAMP1 (red). D. Quantification of Ad5-M1 colocalized with LAMP1. E. Western Blot of Atg5 (top) and actin (bottom) in control and Atg5 siRNA transfected cells. *p<0.05, **p<0.005
using 3-MA, a PI3-kinase inhibitor that inhibits autophagosome formation (Figure 15B). Additionally, knockdown of Atg5 decreased the percent of Ad5-M1 that colocalized with the lysosome marker LAMP1 (Figure 15C and 15D). From these data we conclude that the specific infectivity defect of Ad5-M1 can be abrogated by inhibiting the autophagy pathway.

*Ad membrane rupture activates autophagy during cell entry*

We next asked what step in Ad cell entry activates autophagy. While there are many activators of autophagy, TLR9 signaling and reactive oxygen species are known to activate autophagy (Bertin *et al.* 2008; Huang *et al.*, 2011; Chuang *et al.*, 2012; Ye *et al.*, 2012). These two activation signals were of particular interest to us since TLR9 recognizes the Ad dsDNA and since Ad membrane rupture generates reactive oxygen species. We asked if one, or both, of these signals activates autophagy during Ad cellular entry. The ts1 mutant, which does not induce gal3 puncta formation, did not induce LC3 puncta (Figure 16A and 16B) or LC3-II formation (Figure 16C and 16D) (Maier *et al.*, 2012). This indicated that either viral uncoating leading to TLR9 stimulation or membrane rupture is required for Ad-induced autophagy.

To distinguish between the two possibilities, we asked if the Ad5-M1 infectivity defect was TLR9-dependent. Since inhibition of autophagy restores Ad5-M1 infectivity, if TLR9 stimulation activates autophagy during Ad cell entry, then Ad5-M1 should not have an infectivity defect in cells where TLR9 is absent. To address this question, I infected differentiated THP1 cells that were stably knocked down for TLR9 with shRNA or control shRNA with either Ad5-WTgfp or Ad5-M1gfp and the percent of GFP-
Figure 16: Ad5 membrane rupture induces autophagosome formation. HeLa cells were infected for 30 minutes. A. Cells were fixed and stained for LC3 (red). Virus is in green. B. Quantification of LC3 puncta per cell. C. Western Blot of whole cell lysates probed for LC3 and actin. D. Quantification of Western Blot in C. *p<0.05
positive cells was determined 18-22 hours post infection by flow cytometry. Treatment of the THP1 monocyte cell line with PMA differentiates them to become a macrophage-like cell line (Auwerx, 1991). Knockdown of TLR9 did not affect Ad5-M1 infectivity, as the percent of GFP-positive cells by Ad5-M1 was the same as in control cells (Figure 17). We conclude that the Ad5-M1 infectivity defect is TLR9 independent, and suggested to us that membrane rupture is what activates autophagy during Ad cell entry.

*Galectin-8 restriction of Ad5 infection*

Thurston *et al.* demonstrated that in addition to gal3, gal8 and gal9 were recruited to bacterially ruptured membranes (Thurston *et al.*, 2012). Additionally, NDP52, which binds LC3, is also recruited to ruptured membranes and binds gal8 (Thurston *et al.*, 2012). Knockdown of gal8 reduced LC3 colocalization with *Salmonella* and increased bacterial replication (Thurston *et al.*, 2012). Since we see a similar phenotype with our Ad5-M1 virus having decreased infectivity due to autophagy, and that Ad5 membrane rupture recruits gal3 to form punctate structures, we first asked if gal8 and NDP52 were recruited to Ad5 ruptured membranes. If so, we asked if gal8 and NDP52 colocalization was greater for Ad5-M1 compared to Ad5-WT, similar to the gal3 phenotype we observed. We again used synchronous infections and IFA analysis to address these questions, using IMARIS® to define gal8 punctate structures and colocalization. Indeed, we found that Ad5 infection induced gal8 puncta formation (Figure 18A). Again, there was no difference in the number of gal8 puncta between Ad5-WT and Ad5-M1 infected cells (Figure 18B), suggesting that gal8 can be used as a marker for Ad5 membrane rupture. Similar to gal3, Ad5-M1 colocalized with gal8 (Figure 18C) or NDP52 (Figure
Figure 17: Ad5 induces autophagy in a TLR9-independent manner. PMA-differentiated THP-1 cells were infected with varying concentrations of Ad5-WTgfp or Ad5-M1gfp for 18-24 hours. The percent GFP+ cells was determined by FACS.
Figure 18: Ad5 induces gal8 puncta. HeLa cells were infected with Ad5-WT or Ad5-M1 (green). Cells were fixed and stained for gal8 (red). A. Representative images from 30min post infection. Quantification of the number of gal8 puncta per cell (B.) and percent Ad5 colocalized with gal8 (C.).

Figure 19: Ad5 colocalization with NDP52. HeLa cells were infected with Ad5-WT or Ad5-M1 (green). Cells were fixed and stained for NDP52 (red). A. Representative images from 30min post infection. Bar is 5μm. B. Colocalization of Ad5 with and NDP52. *p<0.01 **p<0.001
19) more than Ad5-WT.

We asked if galectin recruitment to Ad5-ruptured membranes restricted Ad5-M1 infectivity. We first addressed this question by infecting cells with Ad5-WT or Ad5-M1 in the presence of 100mM lactose, which binds N-linked glycans, or sucrose, which does not compete for galectin binding. The percent of GFP-positive cells was determined by flow cytometry. If galectin recruitment to Ad5-ruptured membranes targets them for autophagosomes, then we would expect increased Ad5-M1 infectivity in cells treated with lactose compared to sucrose-treated cells. We saw a three-fold increase in percent of GFP-positive cells for Ad5-M1 infection in the presence of lactose (Figure 20). Increasing the lactose concentration did not restore Ad5-M1 infectivity to Ad5-WT levels (data not shown).

To specifically ask if gal8 targeted Ad5-ruptured endosomes for autophagy degradation, we used siRNA knockdown to gal8 and determined Ad5-WT and Ad5-M1 infectivity, as well as colocalization with LC3 and LAMP1 compared to control siRNA-treated cells. We would expect that if Ad5-M1 infectivity restriction is gal8-dependent, then we would see no Ad5-M1 infectivity defect compared to Ad5-WT in gal8 knockdown cells. We found that gal8 knockdown restored Ad5-M1 specific infectivity to the Ad5-WT level (Figure 21). We did find that gal8 knockdown significantly decreased Ad5-WT infectivity, although the decrease was less than 2-fold, compared to the 5-fold increase we saw with Ad5-M1 infectivity.

To determine if the restoration of Ad5-M1 infectivity during gal8 knockdown was due to the autophagy pathway, we determined colocalization with the autophagosome
Figure 20: Effect of lactose on Ad5 infectivity. HeLa cells were pre-treated with 100mM lactose (solid bars) or sucrose (striped bars) for 2 hours and infected with 10ppc of Ad5-WT or Ad5-M1 in the presence of each sugar. The percent of GFP+ cells was determined 18 hours post infection by FACS.
Figure 21: Gal8-dependent restriction of Ad5-M1 infectivity. HeLa cells were transfected twice with control or gal8 siRNA. Cells were infected with 10 particles per cell of Ad5-WT or Ad5-M1 72 hours after the first transfection. A. The percent of GFP+ cells were determined 18 hours post infection by FACS. B. Whole cell lysates were lysed prior to time of infection and probed with antibodies for gal8 and actin. Densitometry was determined using ImageJ.
marker LC3 and the lysosome marker LAMP1 in control and gal8-siRNA treated cells. We would expect that if the gal8-dependent restriction of Ad5-M1 is due to recruiting autophagosomes to Ad5-ruptured membranes, which ultimately traffics Ad5 to lysosomes, then knockdown of gal8 would decrease Ad5-M1 colocalization with LC3 and LAMP1. While gal8 knockdown had no affect on LC3 colocalization with Ad5-WT, colocalization of Ad5-M1 with LC3 significantly decreased (Figure 22A and 22B). Additionally, Ad5-M1 colocalization with LAMP1 was significantly decreased in gal8 knockdown cells (Figure 22C and 22D). We conclude that gal8 restricts Ad5-M1 infectivity by targeting gal8+ membranes to autophagy.

Although Thurston et al. saw a decrease colocalization of Salmonella with LC3 in the absence of gal8, they did not determine if autophagy was still activated by Salmonella infection in gal8 knockdown cells (Thurston et al., 2012). To address if gal8 was required for autophagosome formation during Ad5 infection, we calculated the number of LC3 puncta per cell in Ad5 infected control and gal8 siRNA treated cells using IMARIS® software. We observed no difference in the number of LC3 puncta per cell between control and gal8-siRNA treated cells (Figure 23). Therefore, although the autophagy pathway is still activated in the absence of gal8, gal8 recruitment of LC3 to Ad-ruptured membranes is needed in order to target these membranes to the autophagy pathway.

*The pVI-PPxY motif decreases antigen presentation*

In addition to serving as a degradative pathway for both cellular components and pathogens, autophagy can lead to increased antigen presentation, as autophagosomes fuse
**Figure 22: Gal8-restriction of Ad5-M1.** HeLa cells were transfected twice with control or gal8 siRNA. Cells were infected with Ad5-WT or Ad5-M1 (green) 72 hours after the first transfection. Cells were fixed and stained for gal8 (red) and LC3 or LAMP1 (blue). Representative images for LC3 staining at 30 minutes post infection (A.) and for LAMP1 at 4 hours post infection (C.). Quantification of Ad5 with LC3 (B.) and LAMP1 (D.)

* p<0.03, ** p<0.001
Figure 23: Ad5 induces autophagosome formation in the absence of gal8. The number of LC3 puncta per cell was determined using IMARIS® software from the experiment in Figure 22.
with MHC-II containing compartments (Levine and Deretic, 2007; Nedjic et al., 2009; Gannage and Munz, 2010; Munz, 2010). Ads are currently used as vaccine vectors, but the current generation vectors induce poor CD4+ T cell responses (Lasaro and Ertl, 2009). Since we see that our Ad5-M1 virus is sequestered into autophagosomes and lysosomes more than the WT virus, we asked if the presence of the pVI-PPxY motif was decreasing antigen presentation. To address this question, we infected mice with equal particles of either Ad5-WT, Ad5-M1 or PBS control. Ten days later the mice were sacrificed and splenocytes were stimulated with either Ad5 capsids or GFP purified from E. coli. T cell activation was measured by IFN gamma ELISPOT. We saw more IFN gamma spot forming units (SFU) in splenocytes stimulated with Ad5 capsids from the Ad5-M1 infected mice compared to Ad5-WT infected mice (Figure 24A), suggesting increased Ad5 capsid antigens during Ad5-M1 infection. We also saw a significant decrease in IFN gamma SFUs in the Ad5-M1 infected mice when the splenocytes were stimulated with purified GFP protein compared to Ad5-WT infected mice (Figure 24A). The gfp is encoded in the Ad genome, confirming a defect in Ad5-M1 infectivity in vivo.

Additionally, we generated a human CD4+ T cell clone to a conserved hexon epitope and incubated these T cells with syngenic APCs that were transduced with either Ad5-WT or Ad5-M1. We measured T cell activation by IFN gamma ELISA. Again, we saw increased IFN gamma levels from the T cells that were incubated with the Ad5-M1 transduced APCs compared to Ad5-WT (Figure 24B), suggesting increased antigen presentation on MHC-II. The increase in antigen presentation was not due to increased MHC-II surface expression or CD86 (which provides costimulation for T cell activation).
Figure 24: The affect of the PPxY motif on antigen presentation. A. Mice were infected with 1010 virus particles of Ad5-WTgfp, Ad5-M1gfp or PBS control. 10 days post infection mice were sacrificed and splenocytes were stimulated with Ad5-luc or GFP purified from E.coli. IFNγ was determined by ELIspot. B. CD4+ T-cell clones recognizing a conserved Ad5 hexon epitope were incubated 3:1 with syngeneic APCs transduced with either control media or Ad5-WT or Ad5-M1 for 24 hrs. IFNgamma secretion was quantified by ELISA. C. Human monocyte-derived dendritic cells were transduced with Ad5-WT or Ad5-M1 for 18 hours. Cell surface expression of HLA-DR or CD86 was assessed by FACS. *p<0.05, **p<0.001, # Not significant
surface levels (Figure 24C). This suggests that the pVI-PPxY motif decreases capsid epitope presentation on MHC-II.
CHAPTER IV
DISCUSSION

Early membrane rupture by Ad5

Although a number of studies indirectly document a membrane lytic event during Ad cell entry, visualizing virus rupture of endosomes and subsequent egress of the virus into the cytoplasm has been elusive. Electron microscopic studies of Ad infected cells have been able to capture the virus at the cell surface, in endosomes or in the cytoplasm, but have been unable to adequately describe Ad crossing a disrupted endosomal membrane (Nakano et al., 2000; Wang et al., 2000; Gastaldelli et al., 2008; Imelli et al., 2009). While a few electron microscopic studies have captured viruses that appear to be in the process of escaping ruptured endosomes, these reports are rare, and electron microscopy studies do not allow for staining for multiple specific proteins or live cell imaging (Morgan et al., 1969; Hoenig et al., 1974). Our studies of identifying gal3 as a marker for Ad rupture of endosomal membranes during cell entry has allowed us to demarcate events during Ad cell entry occurring prior to, during or after endosomal membrane rupture such as capsid uncoating, egress from ruptured endosomes and intracellular trafficking.

Using gal3 as a marker for Ad5 membrane rupture, we have identified that Ad5 membrane rupture occurs within 15 minutes after shifting cells to a temperature (37°C) that allows for endocytosis. Our time course studies support prior details of subgroup C
Ad entry. Early electron microscopy studies suggested that Ads could enter the cells in two ways: either by direct plasma membrane penetration or uptake in a vacuole (Morgan et al., 1969; Brown and Burlingham, 1973). However, neither study could capture a viral particle that was halfway through the cell membrane (Morgan et al., 1969; Brown and Burlingham, 1973). Biochemical studies demonstrated subgroup C Ad membrane rupture by measuring release of $^{51}$Cr from loaded cells after exposure to adenovirus (Seth et al., 1984; Seth et al., 1985a). These studies found that Ad2 could directly rupture the plasma membrane (i.e. $^{51}$Cr release), when the virus was incubated in medium with a pH between 5.5 and 7, with maximum $^{51}$Cr release occurring at pH 6 (Seth et al., 1984; Seth et al., 1985a).

Later studies determined that uncoating begins at the cell surface, as shedding of the Ad fiber after receptor engagement was independent of endocytosis (Nakano et al., 2000). A recent study showed that certain events at the cell surface aid with subsequent uncoating events after endocytosis (Burckhardt et al., 2011). Ad fiber binding to CAR initiates diffusive motions and drifts, while penton base binding to integrins initiates more confined motions in the plasma membrane (Burckhardt et al., 2011). Although the diffusive and confined motions were independent of actin, the drifts were dependent on an intact, dynamic actin cytoskeleton and the actin-dependent myosin-2 motor protein (Burckhardt et al., 2011). Inhibition of the drifts, using CAR-negative cells or pharmacologically, reduced fiber shedding, pVI exposure and Ad infection (Burckhardt et al., 2011). These data suggest that mechanical events at the cell surface through CAR binding significantly contribute to Ad uncoating.
We and others see that pVI becomes exposed for antibody binding 5-10 minutes after endocytosis when the viral particles are still near the periphery of the cell (Wodrich et al., 2010; Burckhardt et al., 2011; Maier et al., 2012). We show that these events lead to membrane rupture near the cell surface (Figure 2). Protein VI exposure occurs prior to gal3 puncta formation (Maier et al., 2012) and less than half of pVI-positive Ad5 virions were EEA1-positive (Figure 3A). We also show that approximately 50% of Ad5 membrane rupture (i.e. gal3-positive) events occur outside of early endosome/EEA1 colocalization (Figure 3B), and very few membrane rupture events occurred in lysosomes (Figure 3C). These data suggest that pVI exposure and Ad5 membrane rupture occurs outside or prior to encountering early endosome compartments.

Our data support the mounting evidence that subgroup C Ads do not require exposure to low pH for uncoating and endosome rupture to occur. Early biochemical studies suggested that capsid exposure to low pH was required for the release of internal capsid proteins and some hexon (Seth et al., 1984, 1985b; Greber et al., 1993; Prchla et al., 1995). Wiethoff et al. saw a 40-fold reduction in Ad5 membrane rupture in cell culture in the presence of the vacuolar H+-ATPase inhibitor, bafilomycin A1 (Wiethoff et al., 2005). However, some direct Ad2 rupture of the plasma membrane can occur at neutral pH (Seth et al., 1984). Additionally, other studies have found that neutralizing pH during Ad infection has no affect on membrane rupture or infectivity (Seth et al., 1984; Rodriguez and Everitt, 1996) or that extremely high concentrations of neutralizing agents needed to be used to see a decrease in Ad infectivity (Varga et al., 1991). Also, many studies that support the lowered pH requirement for Ad membrane rupture use...
membrane vesicles instead of infection of intact cells. For example, studies demonstrated that lowered pH was needed for maximal Ad-rupture of liposomes (Blumenthal et al., 1986; Wiethoff et al., 2005). However, these liposomes did not contain Ad receptors, which were recently shown to initiate Ad5 uncoating (Burckhardt et al., 2011). It is possible that capsid uncoating of Ad5 can be pH dependent, but CAR binding is more efficient, hence why *in vitro* studies lacking receptors reveal a pH-dependent uncoating process.

Although it has been accepted in the literature that subgroup C Ads traffic through early endosomes, these studies have not directly tested when subgroup C Ads rupture membranes. These conclusions are based off of mainly two observations. First, subgroup C Ads colocalize with early endosome markers more than late endosome/lysosome markers (Gastaldelli et al., 2008). Second, studies using mutant Ads that do not uncoat (ts1) or have decreased membrane rupture (mutations in pVI) have higher colocalization with early endosome markers than their wild-type counterpart (Gastaldelli et al., 2008; Moyer et al., 2011). By performing co-staining of Ad5 with gal3 and endocytic markers, we have specifically examined whether Ad5 membrane rupture occurs in known endosomal compartment(s).

We only tested one member of the subgroup C Ads (Ad5), and it would be interesting to test gal3 colocalization and endocytic markers with members of subgroup B viruses, which are thought to traffic to late endosomes prior to endosome escape (Miyazawa et al., 2001; Shayakhmetov et al., 2003). Experiments using markers of late endosomes and lysosomes with gal3 staining would determine if subgroup B viruses
traffic to late endosomes prior to membrane rupture. It is possible that subgroup B Ads rupture membranes prior to encountering late endosomes/lysosomes, but remain associated with the membrane until after acquiring these markers prior to endosome escape. While we have observed Ad5 trafficking in gal3+ structures, we can not discern whether our trafficking occurs in membranes that remained ruptured or have been resealed. Either way, gal3 would be a useful tool to further expand upon the knowledge of the different Ad subgroup trafficking in endosomes.

Early membrane rupture could decrease the Ad5-induced immune response. Subgroup C Ads are known to elicit less of an immune response compared to other Ad subgroups (Sakurai et al., 2010; Barlan et al., 2011a; Teigler et al., 2012). One way Ad5 may dampen the immune response by early membrane rupture is by evading recognition by the pattern recognition receptor (PRR) TLR9, which is found in endosomal compartments. TLR9 recognizes the Ad dsDNA genome, leading to activation of NFκB and transcription of cytokines and innate inflammatory components (Appledorn et al., 2008; Muruve et al., 2008; Barlan et al., 2011a; Barlan et al., 2011b; Perreau et al., 2012). TLR9 colocalizes with markers of late endosomes/lysosomes (Chiang et al., 2012). Additionally, TLR9 must be cleaved for signaling to occur, and this cleavage occurs in late endosomes/lysosomes (Park et al., 2008). By uncoating and rupturing membranes prior to encountering TLR9-containing endosomes, Ad5 would evade TLR9 recognition, decreasing NFκB activation and cytokine production.

Ad membrane rupture activates the NLRP3 inflammasome, required for the secretion of the proinflammatory cytokines IL1β and IL-18 (Muruve et al., 2008; Barlan
Studies demonstrate that the amount of IL1β secretion can be altered depending on the endosome compartment in which Ad membrane rupture occurs. Infection with Ads that bind CD46 and traffic to lysosomes leads to greater IL1β secretion compared to Ad5 (Zaiss et al., 2009b; Barlan et al., 2011a). These Ads released more cathepsin B into the cytosol compared to Ad5 at equal particle numbers (Barlan et al., 2011a). Release of active cathepsin B damages the mitochondrial membrane, generating reactive oxygen species (ROS) a requirement for Ad-induced activation of the NLRP3 inflammasome (McGuire et al., 2011). Rupturing membranes early releases less active cathepsin B into the cytosol, decreasing ROS levels and NLRP3 inflammasome activation. Collectively these data suggest that Ad5 membrane rupture prior to or in early endosomes decreases detection of Ad5 by the innate immune response.

While Ad5 ruptures membranes early, we observed that the virus remains colocalized with gal3 and traffics within these gal3+ membranes. Although we don’t know if these viruses are trafficking in gal3+ membranes that have been resealed, Ad5 can escape from these gal3 puncta. We can only speculate as to why Ad5 traffics within these ruptured endosomes. It is possible that Ad5 must traffic to specific cellular locations to engage additional cellular components required for Ad5 endosome escape. Support for this hypothesis comes from a study by Strunze et al. showing that Ad5-pIX engagement with kinesin aids in Ad5 genome delivery by approximately 2-fold (Strunze et al., 2011). In addition to binding to pIX, kinesin components bound to nuclear pore complex proteins, and this pIX-kinesin-Nup interaction lead to “gutted” Ad5 capsids
located at the cell periphery (Strunze et al., 2011). It is highly likely that at least a portion of Ad5 virions need to engage kinesin at the nuclear envelope for endosome escape, and therefore must traffic in their ruptured endosomes.

**Microtubule interaction importance for Ad5 trafficking**

Multiple studies support microtubule involvement during Ad infection, but few have been able to demarcate which step(s) of Ad entry microtubule engagement occurs. One of the first reports of microtubule involvement during Ad infection was by Dales and Chardonnet using pharmacological inhibitors of microtubule polymerization (Dales and Chardonnet, 1973). Although one inhibitor, vinblastine, delayed Ad infection, addition of a different inhibitor, colchicine, did not inhibit Ad trafficking to the nucleus (Dales and Chardonnet, 1973). Subsequent studies that pharmacologically inhibited polymerization of microtubules with nocodazole showed decreased Ad virion accumulation at the nuclear envelope and Ad infectivity (Miles et al., 1980; Everitt et al., 1990; Suomalainen et al., 1999; Leopold et al., 2000; Mabit et al., 2002). Miles et al. also quantified subgroup C Ad association with microtubules by electron microscopy studies (Miles et al., 1980). At 1 hour and 6 hours post infection, 25-24% of Ad particles were associated with cytoplasmic microtubules (Miles et al., 1980).

Additional support for Ad engagement of microtubules for infection comes from studies which inhibited movement along microtubules by non-pharmacologic methods. Leopold et al. microinjected antibodies to microtubule motor proteins (dynein and kinesin) and assessed Ad translocation to the nucleus (Leopold et al., 2000). Microinjection of an antibody against the dynein 74.1kDa intermediate light chain
prevented Ad accumulation at the nucleus, while antibodies against kinesin did not (Leopold et al., 2000). Additionally, dynein-mediated transport of cargo can be inhibited by overexpressing the dynactin subunit dynamitin, which dislodges dynein from its cargo (Echeverri et al., 1996; Burkhardt et al., 1997; Ahmad et al., 1998). Overexpression of dynamitin severely decreased Ad2 nuclear targeting (Suomalainen et al., 1999). Collectively these studies demonstrate Ad nuclear trafficking requires microtubule motor movement, specifically dynein.

Later studies detailed provided further insight into subgroup C Ad-microtubule interaction(s) by performing pull down studies and determining colocalization of Ad particles with microtubule motor components. Bremner et al. determined that the highest percentage of colocalization with Ad5 was observed with the dynein heavy chain 1 (Bremner et al., 2009). A significant colocalization of Ad5 with the dynein intermediate chain 74.1 and the dynactin subunits p150 and Arp1 were also observed, while less than 25% of Ad colocalized with the dynein light intermediate chain 1 or 2 (Bremner et al., 2009). Immunoprecipitation of Ad from infected cells yielded an interaction with dynein, but not dynactin (Bremner et al., 2009). Pull down studies using hexon protein purified from late stage Ad infection yielded interactions with the dynein intermediate and light intermediate chain subunits (Bremner et al., 2009). Whether there was an interaction between hexon and the dynein heavy chain 1, which 75% of the virus colocalized with, was not determined (Bremner et al., 2009).

Although Bremner et al. provided evidence that the Ad5 capsid interacts with dynein, the interactions between the hexon protein and dynein subunits were pH
dependent (Bremner et al., 2009). Hexon exposed to pH 6.4 weakly pulled down the
dynein light intermediate chain 1, and pull down of the intermediate chain and light
intermediate chain was not seen unless hexon was exposed to a pH of 5.4 or lower
(Bremner et al., 2009). Ad exposure to pH 5.4 or lower would occur in late
endosomes/lysosomes, and we see very few (2%) Ad5 membrane rupture events
occurring in late endosomes/lysosomes, this interaction may not be relevant during Ad5
cell entry. Additionally, over half of Ad5 membrane rupture events occur outside of
early endosomes (Figure 3B). If these virions are rupturing membranes prior to
encountering early endosomes, they may not be exposed to the lower early endosome pH
(pH 6.1-6.2) for the interaction with the dynein light intermediate chain 1 to occur. It is
possible that exposure or interaction with cellular components would allow for
interaction with microtubule motor components in the absence of exposure to low pH.
Additionally, since the exposure to low pH requirement was determined for in vitro
binding, the in vitro conditions might be different than what Ad encounters in vivo, and
low pH would not be a requirement for Ad interaction with microtubule components.

To support the data that the Ad hexon interacts with dynein components, Bremner
et al. overexpressed hexon prior to Ad5 infection in HeLa cells. Overexpression of Ad5
hexon limited Ad5 virion accumulation at the nucleus and decreased Ad5 colocalization
with the dynein heavy chain 1 (Bremner et al., 2009). There was no affect on Ad5
distribution in the cells when the Ad proteins V, VII, X, penton base or 100K protein
were overexpressed (Bremner et al., 2009). Finally, Bremner et al. showed that
antibodies directed to hexon decrease Ad5 colocalization with dynein components and
decrease Ad5 movement and nuclear trafficking (Bremner et al., 2009). Although Bremner et al. concluded that these decreases were due to hexon’s inability to engage microtubule components to traffic to the nucleus, a later study found that antibody-coated Ads are target by TRIM21 for degradation (Mallery et al., 2010). Thus, antibodies directed to hexon most likely are not blocking Ad-microtubule interactions, but instead targeting Ads for degradation, decreasing nuclear trafficking.

In agreement with previous studies, we found that Ad5 accumulation at the nuclear membrane decreases with nocodazole treatment (Figure 7). Contrasting to what Bremner et al. concluded, we find that the pVI-PPxY motif mediates Ad5 microtubule movement towards the nucleus. Wodrich et al. previously provided evidence that the pVI-PPxY motif is required for Ad nuclear targeting (Wodrich et al., 2010). Ad5-M1 accumulation at the MTOC was significantly decreased compared to Ad5-WT (Wodrich et al., 2010). Wodrich et al. also overexpressed RFP-tagged pVI-WT and found that pVI-WT colocalized with tubulin and was mobile (Wodrich et al., 2010). The mobility of pVI-WT was inhibited by nocodazole (Wodrich et al., 2010). In contrast, RFP-tagged pVI-M1 was not mobile and it did not colocalize with tubulin (Wodrich et al., 2010). Here, we find that Ad5-M1 has significantly decreased accumulation at the perinuclear region (within 1.5 μm) compared to Ad5-WT (Figure 7). Although there was no difference between Ad5-WT and Ad5-M1 perinuclear virus at 30 minutes post temperature shift in Figure 7B, other replicates of the experiment yielded a significant decrease in Ad5-M1 perinuclear virus compared to Ad5-WT 30 minutes post temperature shift (data not shown). We attribute the defect in Ad5-M1 nuclear targeting to the virus
not being able to engage microtubules for trafficking, since nocodazole treatment did not further decrease Ad5-M1 perinuclear accumulation (Figure 7B). Bremner et al. did not determine if nuclear targeting of Ad5 virions was altered when overexpressing pVI in cells prior to infection. They excluded pVI as a possible Ad protein involved in microtubule engagement because pVI is released from the capsid prior to endosome escape (Bremner et al., 2009). We have not determined if Ad5-M1 virions exhibit decreased colocalization with dynein and dynactin components compared to Ad5-WT. We also have not determined if pVI-WT can pull down any microtubule motor components, and if so, if there is any difference in the pull down profile with pVI-M1. Further studies are needed to determine which Ad5 protein(s) interact with dynein that allows for Ad5 movement towards the nucleus, and if other interactions work with or independently of the pVI-PPxY motif.

In addition to Ad5 trafficking to the nucleus, we conclude that microtubules are also important for Ad5 endosome escape. Our data agree with limited prior studies which propose that Ad engagement of microtubules occurs after membrane lysis. The first study demonstrated that Ad uncoating was required for microtubule association using the ts1 temperature sensitive mutant. When grown at the permissive temperature (33°C), 25-34% of the virions associated with microtubules at 1 and 6 hours post infection, which was similar to the levels of Ad5 associated with microtubules (Miles et al., 1980). However, when grown at the non-permissive temperature (39°C), 74-94% of the virions at were in lysosomes 1 and 6 hours post infection, while only 2-6% of the virions were associated with microtubules (Miles et al., 1980). These data demonstrate
that Ad association with microtubules requires virion uncoating and subsequent membrane rupture. Another study determined that most motile Ad5 particles were trafficking in neutral environments 30-40 minutes post infection by fluorescence ratio imaging (Leopold et al., 2000). There were no motile Ad5 particles observed trafficking in pH environments lower than pH 6.5 (Leopold et al., 2000). Although Leopold et al. did not determine if motile particles were still associated with intact endosomes or ruptured membranes, they concluded that these particles had already escaped the endosome, since they were not in acidified endosomes (Leopold et al., 2000). An alternative interpretation is that the Ad5 particles had ruptured membranes prior to encountering low pH environments.

My data shows that microtubule recruitment occurs after membrane rupture.

There was no difference in the number of gal3 puncta per cell 30 minutes post infection between DMSO and nocodazole treated cells (Figure 5B and 9B), indicating that Ad5 membrane ruptured and gal3 puncta formation is independent of microtubules. Additionally, there was no significant difference in the percent of Ad5-WT that colocalized with gal3 between DMSO and nocodazole treated cells at 30 minutes post temperature shift (Figure 5C) suggesting microtubule-independent recruitment of gal3 to Ad5 ruptured membranes. However, we saw a significant defect in Ad5-WT endosome escape in nocodazole treated cells at later times post infection, as gal3 colocalization was 3-4-fold higher than cells with no nocodazole (Figure 5B). Additionally, mutation of the pVI-PPxY also decreased endosome escape (Figure 8C and 8E), and nocodazole treatment did not further decrease Ad5-M1 decrease escape (Figure 9C). Collectively,
my results show that Ad5 engages microtubules for Ad5 trafficking to the nucleus and for endosome escape, which is dependent on the pVI-PPxY motif.

Future studies are needed to determine how exactly the pVI-PPxY motif is helping with Ad5 microtubule recruitment. As mentioned previously, we see that Ad5-WT traffics in association with ruptured endosomes towards the nucleus prior to endosome escape. We do not know if Ad5-WT has to traffic within these ruptured endosomes to a different cellular location to encounter machinery needed to endosome escape. It is possible that the pVI-PPxY motif engages microtubules and/or dynein motor components to traffic the virion to that particular location for endosome escape. My data that shows decreased Ad5-M1 accumulation at the nucleus and increased gal3 colocalization compared to Ad5-WT supports this possibility. These data also support the possibility that the pVI-PPxY motif engages dynein for Ad5 trafficking to the nucleus and also is required for engagement of other proteins for endosome escape. Future studies are needed to distinguish between these two possibilities. Identification of cellular proteins involved in Ad5 endosome escape would be of particular interest, and would help determine the mechanism of pVI-PPxY in Ad5 endosome escape.

Although Leopold et al. determined that microinjection of antibodies against kinesin did not alter Ad trafficking to the nucleus, a recent study by Strunze et al. determined a role for kinesin in Ad genome deliver (Leopold et al., 2000; Strunze et al., 2011). They demonstrated kinesin binding to both the Ad capsid protein IX and nuclear pore complex proteins and hypothesized that movement of kinesin away from the nucleus further disassembles the Ad capsid and disrupts the nuclear pore complex (Strunze et al.,
Further disassembly of the Ad capsid and disassembly of the nuclear pore complex increases Ad DNA genome delivery to the nucleus (Strunze et al., 2011). What has not been determined is if Ads engage kinesin and the nuclear pore while they are still associated with endosomal membranes. Deletion of pIX or knockdown of the kinesin light chain 1 decreases Ad specific infectivity by 2-fold (Strunze et al., 2011). In our hands, deletion of pIX from Ad5 displays a 3-fold decrease in specific infectivity (K. McGuire, data not shown), but we have not determined if this mutant virus also has a defect in endosome escape. We also have not determined if Ad5-WT colocalization with gal3 increases with kinesin light chain 1 knockdown. If deletion of pIX or knockdown of kinesin increases gal3 colocalization, it would be interesting to determine the pVI-PPxY motif mediates kinesin recruitment to the Ad5 capsid for pIX binding. To address this possibility, we would measure gal3 colocalization of Ad5 that has pIX deleted and the M1 mutation (Ad5-M1ΔpIX) and compare it to Ad5-M1. If colocalization with gal3 is the same for both viruses, then it would suggest that the pVI-PPxY motif mediates kinesin recruitment to allow for pIX binding. However, if colocalization of Ad5-M1ΔpIX with gal3 is higher than Ad5-M1, it would mean there are two separate mechanisms for Ad5 endosome escape. This might explain why we still observed a decrease in Ad5-M1 infectivity when microtubules were depolymerized with nocodazole (Figure 10).

There are few reports that connect ubiquitination of a particular proteins or E3-ubiquitin ligases to microtubule engagement. Rheb, a GTPase, disrupts dynein-mediated transport of ubiquitinated proteins (Zhou et al., 2009). While Rheb itself can be
ubiquitinated, ubiquitination has only been found to regulate Rheb degradation and not function (Seo et al., 2010). There have been multiple reports on E3-ubiquitin ligases trafficking via microtubules. The microtubule-associated protein MID1 has ubiquitin ligase activity and can migrate along microtubules when it associates with the regulatory subunit of protein phosphatase 2A (PP2A), a protein MID1 ubiquitinates (Aranda-Orgilles et al., 2008).

More definitive evidence of ubiquitin-dependent recruitment of microtubules comes from a study of BCR signaling and antigen gathering (Schnyder et al., 2011). During B cell receptor (BCR) signaling, the E3 ubiquitin ligase Cbl is recruited to the BCR microcluster (Schnyder et al., 2011). Recruitment of Cbl to the BCR allows for BCR interaction with the microtubule motor dynein heavy and light chains and movement along microtubules was observed (Schnyder et al., 2011). Dyenin recruitment to BCR microclusters was impaired in Cbl-/- B cells. However, Cbl did not directly bind dynein components (Schnyder et al., 2011). Syk, ubiquitinated by Cbl, did immunoprecipitate dynein components (Schnyder et al., 2011). Although not directly tested, they proposed that Cbl ubiquitinates Syk in the BCR microcluster, which allows Syk to interact with such components (Schnyder et al., 2011).

Although the MID1 traffics along microtubules and Cbl recruited to the BCR microcluster leads to dynein recruitment, neither of these examples are in the context of a pathogen invading a cell. Although many viruses use microtubules during infection (recently reviewed in (Dodding and Way, 2011), examples of ubiquitination during infection and microtubule engagement are limited. Besides our data with Ad5-pVI, the
closest example found in the literature is the *Autographa californica* nucleopolyhedrovirus capsid protein EXON0, which binds to β-tubulin and kinesin and is required for efficient egress of newly generated viral capsids from the nucleus (Dai *et al.*, 2004; Fang *et al.*, 2007; Fang *et al.*, 2009; Danquah *et al.*, 2012). Interestingly, EXON0 contains a RING finger domain like those found in certain E3 ubiquitin ligases (Dai *et al.*, 2004). Whether the presence of the RING finger in EXON0 is required for kinesin engagement has yet to be tested.

**Evading autophagy by endosome escape**

Decreasing Ad5 endosome escape, by either pharmacological agents or mutation of the pVI-PPxY motif, decreases Ad5 infectivity (Figures 6A, 6B and 10). The consequence of remaining attached with a ruptured endosome membrane is being targeted for degradation in the lysosome via autophagy. We propose that the presence of the pVI-PPxY motif allows Ad5 to escape the endosome before autophagosomes sequester the membrane fragments. If the Ad5-M1 virus has enough time to escape the endosome by inhibiting the autophagy pathway or knocking down gal8, Ad5-M1 infectivity is restored to Ad5-WT levels.

This is the first report, to our knowledge, that Ad5 cell entry activates autophagy. Previous studies did not test autophagy activation in Ad infected cells until at least 12 hours post infection (Jiang *et al.*, 2008; Jiang *et al.*, 2011; Piya *et al.*, 2011; Rodriguez-Rocha *et al.*, 2011). We see membrane rupture initiates autophagy activation within 30 minutes of Ad5 infection (Figures 12 and 16). The Ad-E1B19K protein has been found to interact with Beclin-1, activating autophagy (Piya *et al.*, 2011). Since our Ads do not
contain the E1 region, activation at the early time points of infection must be by another mechanism. We still observed autophagy activation during Ad5 entry in the absence of gal8 (Figure 23), therefore recruitment of gal8 to Ad-ruptured membranes does not activate autophagy within the cell. The generation of ROS by Ad membrane rupture could activate autophagy during entry. ROS production activates autophagy and the ROS scavenger N-acetyl-L-cysteine (NAC) inhibits autophagy induced by starvation or cytokines (Scherz-Shouval and Elazar, 2007; Underwood et al., 2010; Huang et al., 2011; Chuang et al., 2012). However, in our hands we observed decreased specific infectivity for both Ad5-WT and Ad5-M1 in the presence of N-acetyl-cysteine (data not shown). We, therefore, did not determine the autophagy status of NAC-treated cells. The exact mechanism(s) for autophagy activation during Ad cell entry remains to be determined.

Inhibiting autophagy by Atg5 siRNA knockdown did not affect Ad5-WT infectivity (Figure 15A), but we did see a significant decrease of Ad5-WT infectivity in gal8 siRNA knockdown cells (Figure 21A). We do not know the exact reason why Ad5-WT infectivity decreased, though gal8 and other galectins can bind to integrins, which Ads use for cell entry and endocytosis (Carcamo et al., 2006; Friedrichs et al., 2008; Lei et al., 2009). Knockdown of gal3 alters integrin expression levels, but whether knockdown of gal8 has the same effect has not been determined (Carcamo et al., 2006; Friedrichs et al., 2008; Lei et al., 2009). Additionally, gal8 knockdown decreases endocytosis of platelet coagulation factor V in megakaryocytes (platelet precursor cells) (Zappelli et al., 2012). It is possible that knockdown of gal8 alters Ad5 endocytosis in HeLa cells, decreasing Ad5-WT infectivity in our experiments. Either way, we believe
that the affect that gal8 knockdown on has on Ad5-WT infectivity would affect Ad5-M1 to a similar degree. We saw a similar phenotype in gal3 or NDP52 siRNA knockdown in HeLa cells. Knock down of either protein significantly decreased Ad5-WT infectivity, while Ad5-M1 infection (as measured by percent gfp+ cells) was unchanged compared to control siRNA treated cells (data not shown).

We conclude that gal8 recruitment of autophagy machinery to the ruptured membranes reduces Ad5-M1 infectivity. We saw reduced colocalization of the autophagosome marker LC3 with Ad5-M1 in the absence of gal8, whereas Ad5-WT colocalization was unaffected (Figure 22B). Gal8 knockdown had the same effect on Ad5-M1 colocalization with LAMP1 (Figure 22D). We would presume a model that gal8 recruitment to Ad-ruptured membranes recruits NDP52, which would bind gal8 and LC3. Thurston et al. found less NDP52 recruited to Salmonella when gal8 was knocked down (Thurston et al., 2012). We did not stain for NDP52 in our gal8 knockdown cells, but we assume that gal8 works through a similar mechanism. However, the same study also found that NDP52 is recruited in two phases: one gal8-dependent and one gal8-independent, ubiquitin-binding dependent (Thurston et al., 2012). The gal8-independent mechanism is recruited to Salmonella at later times during infection (4 hours) compared to gal8-mediated recruitment of NDP52 to Salmonella at 1 hour post infection (Thurston et al., 2012). Although we did not directly test if Ad5 ruptured membranes recruits NDP52 by the same two independent mechanisms, we would hypothesize that this later, gal8-independent recruitment a minimal affect on Ad5 targeting. First, the colocalization of Ad5-M1 with LC3 is at its highest 30 minutes post infection. Second, the
colocalization of Ad5-M1 with LAMP1 is restored to Ad5-WT levels at 1 hour post infection. These two results indicate that autophagosomes are quickly formed around Ad5-ruptured membranes, instead of at later times when gal8-independent NDP52 recruitment would occur. Finally, Ad5-M1 infectivity is restored to Ad5-WT levels in gal8 knocked down cells. I measured Ad infectivity not less than 14 hours post infection. If Ad5-M1 remained in endosomes at 4 hours post infection, when gal8-independent NDP52 recruitment occurs, then we would see no restoration of Ad5-M1 infectivity due to LC3 recruitment by NDP52 at later time points.

From my data, we propose the following model of Ad5-pVI-PPxY motif during cell entry detailed in Figure 25. Soon after Ad5 endocytosis, pVI becomes exposed and released from the capsid, rupturing membranes. Membrane rupture activates autophagy in the cells. Exposure of N-linked glycans by Ad5 membrane rupture recruits galectins 3 and 8, as well as the autophagy receptors p62 and NDP52, and gal8 recruitment ultimately recruits the autophagosome marker LC3. Meanwhile, the pVI-PPxY motif aids in Ad-engagement of microtubules to traffic to the nucleus and for Ad5 endosomal escape.

Whether the PPxY motif simply is involved in Ad5 trafficking to the appropriate spot where the machinery needed for endosome escape is located, or if the PPxY motif performs a second function for endosome escape is yet to be determined. If Ad5 does not escape the endosome fast enough, autophagy sequesters the virus associated with the ruptured membrane, shuttling them to lysosomes for degradation. In the absence of the pVI-PPxY motif, if either gal8 is absent, autophagy is inhibited all together, the virus is given enough time to eventually deliver its genome to the nucleus. Whether this is
Figure 25: Model for the pVI-PPxY motif during Ad5 cell entry. Soon after Ad5 endocytosis, pVI becomes exposed and is released from the capsid, rupturing membranes. Membrane rupture activates autophagy in the cells. Exposure of N-linked glycans by Ad5 membrane rupture recruits galectins 3 and 8, as well as the autophagy receptors p62 and NDP52 (not shown), and gal8 recruitment ultimately recruits the autophagosome marker LC3 (green membrane). Meanwhile, the pVI-PPxY motif aids in Ad-engagement of microtubules to traffic to the nucleus and for Ad5 endosomal escape.
achieved by a slower recruitment of the same mechanism, or a separate mechanism all together is not yet known.

More specific experiments are needed to further define how the pVI-PPxY motif is working during Ad5 cell entry. Wodrich et al. showed that the pVI-PPxY motif recruits Nedd4 E3 ubiquitin ligases and that knockdown of Nedd4.2 decreases Ad5-WT infectivity (Wodrich et al., 2010), whereas knockdown of Nedd4.2 did not decrease Ad5-M1 infectivity (H. Wodrich, personal communication with C. Wiethoff). In addition to recruiting Nedd4.2, the pVI-PPxY motif could also be activating Nedd4.2 during infection. Some Nedd4 family members exist in an autoinhibited state (Gao et al., 2004; Gallagher et al., 2006; Wiesner et al., 2007). Although the details of activation are still poorly understood, Nedd4 family-interacting proteins (NDFIPs) activate HECT-E3 ubiquitin ligases by binding to them (Gallagher et al., 2006; Wiesner et al., 2007; Mund and Pelham, 2009). NDFIPs are small transmembrane proteins and contain PY motifs (PPxY, PPLP) in their cytoplasmic tails (Konstas et al., 2002). Mund and Pelham proposed that HECT-E3 ubiquitin ligases are bound to themselves in a conformation that does not allow them to access their substrates and ubiquitinate them (Mund and Pelham, 2009). Binding of NDFIPs to these ubiquitin ligases promotes ubiquitination of substrates, possibly by altering the E3’s conformation to allow them to access their substrates (Mund and Pelham, 2009).

NDFIPs are found localized to the Golgi, multivesicular bodies, and endosomes (Harvey et al., 2002b; Konstas et al., 2002; Cristillo et al., 2003; Shearwin-Whyatt et al., 2004). Shearwin-Whyatt determined that most of a particular NDFIP (N4WBP5A) is
localized in late endosomes (Shearwin-Whyatt et al., 2004). NDFIP2 also localized in punctate structures, suggestive of endosomes (Mund and Pelham, 2009). When the PY motifs of NDFIP2 were mutated, Nedd4 was diffuse throughout the cytosol, compared to colocalizing with NDFIP2 in punctate structures (Mund and Pelham, 2009). NDFIP2 induced ubiquitination by Nedd4, which was dependent on the PY motifs (Mund and Pelham, 2009). It is possible that in addition to recruiting Nedd4, the pVI-PPxY motif is serving as an NDIFP, activating Nedd4 to ubiquitinate substrate(s) needed for Ad5 trafficking and endosome escape.

While our data indicates the involvement of the Ad5-pVI-PPxY motif in Ad trafficking towards the nucleus, endosome escape and evasion of autophagy, the pVI-PPxY motif has recently be implicated in Ad transcriptional activation (Schreiner et al., 2012). Using replication competent Ad5s, Schreiner et al. saw decreased early Ad gene products in Ad5-M1 infected cells compared to Ad5-WT infected cells (Schreiner et al., 2012). The cells were infected with equal fluorescence focus units of replication competent Ad5-WT and Ad5-M1 to overcome the specific infectivity defect of Ad5-M1 (Schreiner et al., 2012). Transcription initiation by Ad5-M1 was dose dependent, as there was no difference in Ad5 E2 promoter activity between Ad5-WT and Ad5-M1 infected cells at high particle per cell ratios (Schreiner et al., 2012). PVI-WT interacted with the transcriptional repressor Daxx, in a PPxY-dependent manner, promoting transcription of the Ad5 E1 promoter (Schreiner et al., 2012). I measured Ad5 infectivity by GFP fluorescence and the GFP cassette is driven by a CMV promoter. It is highly possible that the pVI-PPxY motif plays multiple roles in Ad5 infection.
Mutation of the pVI-PPxY motif: A better vaccine vector?

Ad-based vectors are attractive candidates as vaccine vectors for a number of reasons. The viral genome is readily manipulated; they achieve high levels of transgene expression \textit{in vivo}; they can infect a broad range of target cells; and replication-defective Ads can be propagated in cell culture (Matthews, 2011). The pVI-PPxY motif increases Ad5 genome delivery, which would increase transgene expression of a particular peptide and generate a potent CD8$^+$ T cell response. However, Ad vaccine vectors are poor inducers of CD4$^+$ T cell responses, and recent studies have focused on increasing the CD4$^+$ T cell and antibody response to pathogen peptides incorporated into the Ad vector. These studies have inserted pathogen peptides onto Ad5 capsid proteins (such as hexon or pIX) and have shown increased CD4$^+$ T cell responses and antibody titers to that peptide compared to inserting the gene for the antigen into the Ad5 genome (Parks, 2005; Matthews \textit{et al.}, 2008; Bayer \textit{et al.}, 2010; Matthews, 2011).

We show that the pVI-PPxY decreases Ad5 capsid antigen presentation on MHC-II for CD4$^+$ T cell activation (Figure 24). Ad5-M1 infection activated CD4$^+$ T cells generated against an Ad5 capsid antigen more than Ad5-WT (Figure 24B). There was no difference in APC activation or cellular surface levels of MHC-II between Ad5-WT and Ad-M1 infected cells (Figure 25C). We attribute the increase in CD4$^+$ T cell activate to increased Ad5 capsid loading on MHC-II for the Ad5-M1 virus since the virus is targeted to autophagosomes and lysosomes more than Ad5-WT. We did not test if the increased CD4$^+$ T cell activation for Ad5-M1 capsid antigens was required the autophagy pathway. We would expect that inhibition of the autophagy pathway would abrogate the increased
CD4+ T cell activation in Ad5-M1 infection, which has been observed previously with other viral proteins. Inhibition of autophagy decreases CD4+ T cell recognition of Epstein Barr virus protein EBNA1 without changing MHC-II surface levels (Paludan et al., 2005).

It would be interesting to see if inserting pathogen peptide(s) in the hexon or pIX in the Ad5-M1 virus would further increase antigen presentation of that peptide. Other Ad serotypes have shown to induce a great immune and vaccine responses compared to Ad5, and provide better protection against subsequent challenge of certain pathogens (Geisbert et al., 2011). Since preexisting immunity is another major limitation of using Ad5 as a vaccine vector (Lasaro and Ertl, 2009), it would be interesting to see if mutation of the PPxY motif into these other Ad serotypes would produce an even better immune response and protection.

**Additional pVI regions involved in Ad cell entry**

Although the PPxY motif is conserved across all the sequence human Ad serotypes, the surrounding sequences surrounding the motif vary greatly across human subgroups. The PPxY motif in Ad5 pVI is part of a PEST sequences (areas heavy in proline, glutamic acid, serine, and threonine, indicated in red in Figure 26). Ad5 contains an additional PEST sequence just upstream of the PPxY-containing PEST sequence. Subgroups A and G do not contain a second PEST upstream of the PPxY-containing PEST, while subgroup E Ads have a second PEST sequence located downstream of the PPxY motif. Subgroup F Ads contain the PPxY motif, but no PEST sequences.

PEST sequences are found in many short lived proteins and are thought to serve
as signals for degradation. Mutation of the PEST sequence in specific proteins increases that protein’s half-life (Tsurumi et al., 1995; Marchal et al., 1998; Spencer et al., 2004; Schnupf et al., 2006). Additionally, adding a PEST sequence to a relatively stable protein decreases that protein’s half life (Rechsteiner and Rogers, 1996). PEST sequences within proteins have been found to have other functions, such as transcription regulation or virulence (Lety et al., 2001; Schnupf et al., 2006; Sue and Dyson, 2009).

An overwhelming amount of evidence supports PEST sequences target proteins for degradation via the ubiquitin-proteasome pathway (Rechsteiner and Rogers, 1996; Marchal et al., 1998; Spencer et al., 2004; Schnupf et al., 2006; Xing et al., 2010). PEST sequences can recruit and interact with E3 ubiquitin ligases to mediate degradation (Xing et al., 2010). However, Eto et al. demonstrated that the PEST sequence in nuclear protein kinase C regulates calpain-mediated degradation, suggesting that PEST sequences can be regulated by other pathways (Eto et al., 1995). PEST sequences also interact with other proteins to regulate their function. For example, the IkBα PEST sequence interacts with the DNA binding pocket of NFκB when NFκB is inactive (Sue and Dyson, 2009).

Although mutating the pVI-PPxY motif abolishes Nedd4 recruitment (Wodrich et al., 2010), it would be interesting to determine if the PEST sequence(s) surrounding the PPxY motif also influence E3 ligase recruitment. Is the PEST sequence surrounding the PPxY motif also required for Nedd4 recruitment? Are proteins other than E3 ligases involved? Since the sequence surrounding the PPxY motif vary between subgroups, it would be interesting to determine E3 ligase recruitment, Ad endosomal escape and how this region of pVI influences Ad infectivity.
Figure 26: PEST sequences within pVI. Alignment of the pVI-PPxY motif (black box) in the different Ad subgroups. Sequences rich in proline, glutamic acid, serine and threonine (PEST) sequences are shown in red as determined by ePESTfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind).
Conclusion

Overall, our study has identified an additional immune defense activated during Ad5 cell entry, autophagy. We have also determined how Ad5 evades this immune response. By conservation of the pVI-PPxY motif, Ad5 efficiently escapes endosomes, avoiding sequestration into autophagosome and ultimate delivery to lysosomes. Future studies will determine how the pVI-PPxY motif aids in Ad5 endosome escape and if infection with Ad5-M1 increases MHC-II presentation of capsid-displayed antigenic peptides that are inserted into the Ad capsid. Mutation of the pVI-PPxY motif in other serotypes of Ad, where preexisting immunity to those serotypes is not as prevalent, could render it a better vaccine vector.
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