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## Emerging roles for ubiquitin in adenovirus cell entry

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### Abstract

Adenovirus relies on numerous interactions between viral and host cell proteins to efficiently enter cells. Undoubtedly, post-translational modifications of host and cellular proteins can impact the efficiency of this cell entry process. Ubiquitylation, once simply thought of as a modification targeting proteins for proteasomal degradation, is now known to regulate protein trafficking within cells, protein-protein interactions, and cell signaling pathways. Accumulating evidence suggests that protein ubiquitylation can influence all stages of the life cycle of other viruses such as cell entry, replication, and egress. Until recently the influence of ubiquitylation has only been documented during adenovirus replication. This review highlights the most recent evidence demonstrating direct engagement of host ubiquitylation and SUMOylation machinery by adenovirus during cell entry. Additionally, potential roles for host protein ubiquitylation and the potential for adenovirus regulation of host ubiquitylation machinery during cell entry are discussed.

### INTRODUCTION

From cell attachment to evading the host immune system, adenoviruses must efficiently utilize their ~40-encoded proteins to productively infect cells. Twelve of these virally encoded proteins are present in adenovirus virions (Figure 1), each potentially playing an important role in mediating virus entry into host cells (Nemerow *et al.*, 2009; Russell, 2009) or establishing a niche in the nucleus for viral gene transcription and replication (Chen *et al.*, 2007; Hindley *et al.*, 2007; Karen and Hearing, 2011). Adenovirus, like all viruses, has evolved strategies to usurp host cell molecules for their own benefit. While several well characterized interactions between virion structural proteins and host cell factors have been documented as the virus enters cells (Nemerow *et al.*, 2009), recent reports suggest that there are virus-host interactions during adenovirus cell entry which are still poorly characterized. Furthermore, both virus and host may have evolved strategies to manipulate these interactions between host and viral proteins through a variety of post-translational modifications.

Ubiquitylation of viral and host proteins can influence a variety of processes in virus entry, replication and egress. While numerous reports have highlighted the interaction of adenovirus proteins with the ubiquitin/proteasome system (UPS) during viral replication (Querido *et al.*, 2001; Harada *et al.*, 2002; Blanchette *et al.*, 2004; Woo and Berk, 2007; Schwartz *et al.*, 2008; Isobe *et al.*, 2009; Blackford *et al.*, 2010), recent evidence suggests that ubiquitylation of adenovirus structural proteins and host cell proteins can influence the cell entry of adenoviruses (Gout *et al.*, 2010; Wodrich *et al.*, 2010). A greater understanding

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of the influence of the UPS in adenovirus cell entry is likely to identify novel mechanisms which viruses use to enter cells and may also identify new targets for antiviral therapeutics. This review will highlight recent observations implicating ubiquitin as an important posttranslational modification of host and cellular proteins during adenovirus cell entry. Interactions of adenovirus structural proteins with host ubiquitylation machinery and potential regulation of adenovirus receptors by the ubiquitin modifications will be discussed.

### Adenovirus Cell Entry

Adenoviruses (Ads) are non-enveloped, double-stranded DNA viruses that cause upper respiratory tract, gastrointestinal tract and ocular infections. There are >50 human serotypes, which are classified into 7 subgroups (A–G) (Aoki *et al.*, 2011; Seto *et al.*, 2011). Human Ads bind to cells via an interaction between the adenovirus fiber protein and a primary attachment receptor. Subgroups A, E and F use the Coxsackievirus 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; Dehecchi *et al.*, 2001). Subgroup B viruses use CD46, HSPG, CD80/86, and Densin 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 (Shayakhmetov *et al.*, 2005; Parker *et al.*, 2006; Alba *et al.*, 2009).

After attachment, an interaction between the adenovirus penton base and  $\alpha_v$  integrins triggers clathrin-mediated endocytosis (Nemerow and Stewart, 1999; Smith *et al.*, 2010) and likely initiates stepwise disassembly of the adenovirus capsid (Greber *et al.*, 1993), a process necessary for adenovirus escape from endosomal compartments (Greber *et al.*, 1993; Wiethoff *et al.*, 2005). 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 prior to endosomal escape (Miyazawa *et al.*, 2001; Shayakhmetov *et al.*, 2003). Within endosomes, the adenovirus capsid partially uncoats, a process necessary for adenovirus rupture of endosomal membranes to access the cytoplasm. Partial capsid disassembly is required to release an internal capsid protein, protein VI (pVI), which was recently shown to rupture endosomal membranes, allowing the partially disassembled virion to enter the cytoplasm (Wiethoff *et al.*, 2005; Maier *et al.*, 2010; Moyer *et al.*, 2011). After endosome escape, adenovirus traffics to the nucleus via microtubules, docking at the nuclear pore and delivers the adenovirus genome to the nucleus. Several groups have suggested that determinants in the major capsid protein, hexon (Smith *et al.*, 2008; Bremner *et al.*, 2009; Strunze *et al.*, 2011), and pVI (Wodrich *et al.*, 2010) influence microtubule-dependent trafficking of adenovirus virions in the cytoplasm. Docking of partially disassembled adenovirus virions at the nuclear envelope occurs at nuclear pore complexes. 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).

### The Ubiquitin Proteasome System (UPS)

Ubiquitin plays essential roles in multiple cellular processes. Ubiquitin is conjugated to proteins via amide formation between the ubiquitin C-terminus and  $\epsilon$ -amino groups on lysine side chains of proteins (Kerscher *et al.*, 2006). Once a ubiquitin is appended to a given protein, subsequent ubiquitin molecules can be either conjugated to another lysine

residue of the same protein (multiubiquitylation) or appended to one of seven lysine residues on ubiquitin itself (polyubiquitylation) (Kim and Rao, 2006). Conjugation of ubiquitin to proteins relies on the activity of three classes of enzymes termed E1, E2, and E3-ubiquitin ligases. It is the E3-ubiquitin ligase which provides the substrate specificity and ultimately conjugates ubiquitin to the target protein (Nagy and Dikic, 2010). While protein ubiquitylation 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-ubiquitylation in protein trafficking and activity and the assembly of signaling complexes (Kim and Rao, 2006). Protein ubiquitylation is highly dynamic, with possibly hundreds of E3 ubiquitin ligases and approximately 100 deubiquitinases (Nijman *et al.*, 2005) tightly regulating ubiquitin modifications of proteins. Numerous examples have highlighted the influence of the 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.

## ADENOVIRUS CAPSID PROTEINS AND UBIQUITYLATION

### 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). Present as ~342 copies in the adenovirus virion, protein VI is released from the capsid interior during partial disassembly of the virus within endosomes. Protein VI was recently found to possess all of the *in vitro* membrane disrupting activity of the adenovirus capsid and antibody neutralization (Maier *et al.*, 2010) or genetic impairment of pVI membrane lytic activity (Moyer *et al.*, 2011) were recently shown to prevent adenovirus membrane rupture. 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). PPxY motifs are recognized by WW-domains, commonly found in HECT-domain E3-ubiquitin ligases of the Nedd4 family (Ingham *et al.*, 2004). Recruitment of these Nedd4 family members frequently leads to the ubiquitylation of the PPxY-containing substrate or associated proteins (Sudol, 1996; Harty *et al.*, 2000). *In vitro* studies demonstrated that protein VI is oligo-ubiquitylated (2–3 ubiquitins) by a cytosolic factor and that this ubiquitylation 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.*, 2010). 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.

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, when penton base is overexpressed in cells in culture, it is able to recruit 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 (Weber, 1976). It has also been demonstrated that Avp's protease activity is required for efficient adenovirus cell entry (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 deubiquitylating and deISGylating activity, which has been observed *in vitro* and *in vivo* (Balakirev *et al.*, 2002). While deubiquitylating activity was

observed after 12 hrs during infection based on global reduction in the number of ubiquitylated proteins observed in western blots, the deubiquitylating 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 Avp deubiquitylation of one or several host or viral proteins during adenovirus entry is required for efficient intracellular trafficking to the nucleus.

## UBIQUITYLATION AND ADENOVIRUS RECEPTORS

Adenovirus relies on cell surface receptors for cell attachment to initiate infection. Secondary engagement of  $\alpha_v$  integrins after attachment initiates viral endocytosis. The receptors used by adenovirus are known to influence not only cell tropism (Sharma *et al.*, 2009), but also the intracellular fate of the virus (Wickham *et al.*, 1994; Miyazawa *et al.*, 1999; Carey *et al.*, 2007). A better understanding of how adenovirus receptor cell surface expression and intracellular fate are regulated could improve our understanding of adenovirus pathogenesis. Additionally, since mechanisms controlling adenovirus receptor cell surface expression and intracellular fate could be dysregulated in certain disease states, a better understanding of this process could improve our ability to select appropriate adenovirus vectors for gene therapy.

One potential mechanism influencing adenovirus receptor biology is the UPS. Previous reports demonstrate receptor interactions with UPS machinery during pathogen entry (Khor *et al.*, 2003; Abrami *et al.*, 2006; Coller *et al.*, 2009). Tumor endothelial marker 8 (TEM8), a receptor for *Bacillus anthracis* lethal toxin, is ubiquitylated within 10 minutes of toxin treatment (Abrami *et al.*, 2006). Mutating one of the 16 lysine residues greatly diminished TEM8 ubiquitylation, which lead to a delay in TEM8 endocytosis in response to toxin treatment, indicating that ubiquitylation of TEM8 is necessary for efficient endocytosis (Abrami *et al.*, 2006). TEM8 ubiquitylation (and subsequent internalization) depended on the Cbl E3 ubiquitin ligase, as knockdown of Cbl abolished toxin-induced TEM8 ubiquitylation 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 ubiquitylation may influence internalization.

Additionally, influenza virus requires ESCRT machinery for transit of viral ribonucleoproteins (vRNPs) through the endocytic network and into the nucleus (Khor *et al.*, 2003). ESCRT machinery recognizes membrane-bound ubiquitylated 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, ubiquitylation 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 ubiquitylation. 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 ubiquitylation. 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 ubiquitylation 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 ubiquitylation sites. Lack of the cytoplasmic tail could prevent ubiquitin-dependent downregulation of CAR from the cell surface. Alternatively, if CAR is ubiquitylated in its cytoplasmic tail, ubiquitylation may serve as a signal to target CAR for endocytosis and trafficking to lysosomes for degradation. Deleting the cytoplasmic tail, and potentially abolishing ubiquitylation, might allow CAR to be recycled back to the cell surface.

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 be examined. Second, a role for ubiquitylation 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. Although not directly addressed by Excoffon *et al.* it would be interesting to determine whether MAGI-1b dependent degradation of CAREx8 involves ubiquitylation 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 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 can 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 ubiquitylation, down-regulation, and lysosomal targeting of receptors. Interestingly, CD46 is down-regulated after measles infection, 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 ubiquitylation status of CD46, and if Cbl ubiquitylates CD46, have 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 ubiquitylation in the regulation of CD46- endocytosis and trafficking during adenovirus cell entry.

### $\alpha_v$ 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  $\alpha_v\beta_3$ ,  $\alpha_v\beta_3$  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.*, 2010). Interestingly, several observations suggest that the intracellular trafficking of adenovirus is greatly influenced by the  $\alpha_v$  integrin used by the virus for endocytosis (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 ubiquitylation 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  $\alpha_v\beta_3$  via an RGD motif (Tsai *et al.*, 2008). Additionally, the cell surface levels of  $\alpha_v\beta_3$  were decreased after SPE-B binding, and surface levels were restored with treatment of the proteasome inhibitor MG132, suggesting that  $\alpha_v\beta_3$  internalization and, perhaps recycling is regulated by the ubiquitin-proteasome system (Tsai *et al.*, 2008).

Second, Thomas *et al.* demonstrated that  $\alpha_v\beta_3$  integrin is ubiquitylated in human umbilical vein endothelial cells in response to angiopoietin-2, although whether the alpha or beta chain is ubiquitylated was not described (Thomas *et al.*, 2010). They show that  $\alpha_v\beta_3$  integrin is internalized and ubiquitylated after 10 minutes of angiopoietin-2 ligand treatment (Thomas *et al.*, 2010). No ubiquitylation of  $\alpha_v\beta_3$  integrin was detected in the absence of angiopoietin-2 treatment, demonstrating that ubiquitylation of  $\alpha_v\beta_3$  integrin requires ligand binding. However, it was not determined if  $\alpha_v\beta_3$  integrin was ubiquitylated at the cell surface, resulting in receptor internalization or if ubiquitylation occurred post internalization. Additionally,  $\alpha_v\beta_3$  integrin colocalized with the lysosome marker LAMP1 after 1 hour of angiopoietin-2 treatment, but whether  $\alpha_v\beta_3$  integrin trafficking to lysosomes required ubiquitylation was not determined (Thomas *et al.*, 2010).

To date, no studies determining if  $\alpha_v\beta_5$  integrin is ubiquitylated upon ligand binding have been performed. Initial reports demonstrated that  $\alpha_v\beta_5$  can more greatly support Ad5 infection and endosomal escape than  $\alpha_v\beta_3$  integrins (Wickham *et al.*, 1994). Subsequent studies found that reduced expression of  $\beta_5$  integrin leads to greater targeting of Ad5 to lysosomes (Carey *et al.*, 2007). Although, Carey *et al.* did not determine which integrin  $\beta$ -subunit was utilized in cells with lower  $\beta_5$  expression. Nonetheless, it would be interesting to determine whether differences in the ubiquitylation of  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin are observed

during Ad5 cell entry and whether integrin ubiquitylation correlates with lysosomal trafficking of Ad5.

## SUMOylation

The small ubiquitin-like modifier (SUMO) family of proteins are appended to proteins in a manner similar to ubiquitin and other ubiquitin-like proteins (reviewed in (Geiss-Friedlander and Melchior, 2007)). SUMOylation is another post-translational modification that has recently been shown to influence early events during viral infection. Like ubiquitylation, SUMOylation uses E1, E2, and E3 conjugating enzymes to attach a SUMO to  $\epsilon$ -amino groups of lysine residues, though these SUMO conjugating enzymes are different cellular enzymes than those used for ubiquitylation. Protein SUMOylation has been reported to alter protein localization, activity, stability, and promote protein-protein interactions (reviewed in (Geiss-Friedlander and Melchior, 2007)). Although the SUMOylation occurs with great frequency in the nucleus, recent literature suggests that SUMOylation can regulate protein function and trafficking in the cytoplasm as well (reviewed in (Geiss-Friedlander and Melchior, 2007)). SUMOylation during viral infection has been reported to promote an antiviral defense, or conversely, promote viral infection (reviewed in (Wimmer *et al.*, 2011)). SUMOylation of HIV integrase protein promotes infection at a step following nuclear entry and prior to integration (Zamborlini *et al.*, 2011). SUMOylation of the Moloney murine leukemia virus capsid protein is required for an early step during viral infection after reverse transcription prior to nuclear entry (Yueh *et al.*, 2006). Although the actual role for SUMOylation at these early stages of retroviral infection are incompletely defined, these data are in line with previous observations that SUMOylation regulates nuclear import of number of cellular proteins (Pichler and Melchior, 2002; Lin *et al.*, 2003; Melchior *et al.*, 2003) or the function of nuclear proteins (reviewed in (Geiss-Friedlander and Melchior, 2007)).

While a role for SUMOylation in the organization of Ad replication centers in the nucleus has been documented (Wimmer *et al.*, 2010), no evidence is available to suggest a role for SUMOylation during Ad cell entry. Nonetheless, one recent report has implicated a role for the nuclear pore-associated SUMO E3 ligase, Nup358/RanBP-2 in facilitating nuclear import of Ad2/5 (Strunze *et al.*, 2011). Nup358/RanBP-2 is required for the import of numerous cellular proteins into the nucleus independent of its sumoylating activity (Walde *et al.*, 2011). Nup358/RanBP-2 was also identified as a cell factor that promotes nuclear import of the HIV pre-integration complex (Brass *et al.*, 2008). Strunze *et al.* demonstrated that knockdown of Nup358/RanBP-2 had no effect on Ad localization to nuclear pore complexes, but did decrease Ad2/5 capsid uncoating and reduced Ad-mediated GFP expression (Strunze *et al.*, 2011). They also determined that Nup358/RanBP-2 anchors the Ad2/5 capsid to microtubules through interactions with Nup214-Ad2/5 complexes and kinesin. (Strunze *et al.*, 2011). From these data they concluded that Nup358/RanBP-2 serves as a scaffold protein to link Ad to kinesin, which is important for Ad uncoating and genome delivery. Whether or not the sumoylating activity of Nup358/RanBP-2 is required for Ad uncoating, or if the Ad capsid is sumoylated remains to be examined.

## CONCLUDING REMARKS

Here we have summarized recent observations which suggest that the UPS may influence adenovirus cell entry. These preliminary observations raise many important questions regarding the contributions of ubiquitin to the adenovirus cell entry process. What is the role of recruitment of the Nedd4 family members of E3 ubiquitin ligases during adenovirus infection? It has previously been determined that mutating the motif in pVI responsible for Nedd4 family recruitment decreases adenovirus entry. Would mutating the motif in the penton base also decrease adenovirus entry, and if so, since the penton base and pVI recruit



the same family of E3-ubiquitin ligases, would mutations of the motifs in both proteins further decrease adenovirus entry? Additionally, how does the viral protease, which contains deubiquitylating capabilities, contribute to adenovirus entry? Is deubiquitylating by the viral protease essential for adenovirus cell entry, and if so, what protein(s) is the protease targeting?

Additionally, recent observations suggest that ubiquitylation of adenovirus receptors can regulate their cell surface expression and intracellular trafficking, although the importance of this regulation during adenovirus cell entry is unexplored. Are cellular receptors utilized by adenovirus for entry ubiquitylated after adenovirus attachment, and if so, how does this affect adenovirus transduction? Does ubiquitylation of these cellular receptors result in downregulation and degradation, which might decrease adenovirus transduction? Additionally, does ubiquitylation of these cellular receptors promote signaling to cellular pathways that are essential to adenovirus entry? Is Ad intracellular trafficking influenced by viral or host protein SUMOylation? Answers to these questions should shed considerable light on the biology of adenoviruses and may provide useful insight into novel targets for antiviral therapeutics.

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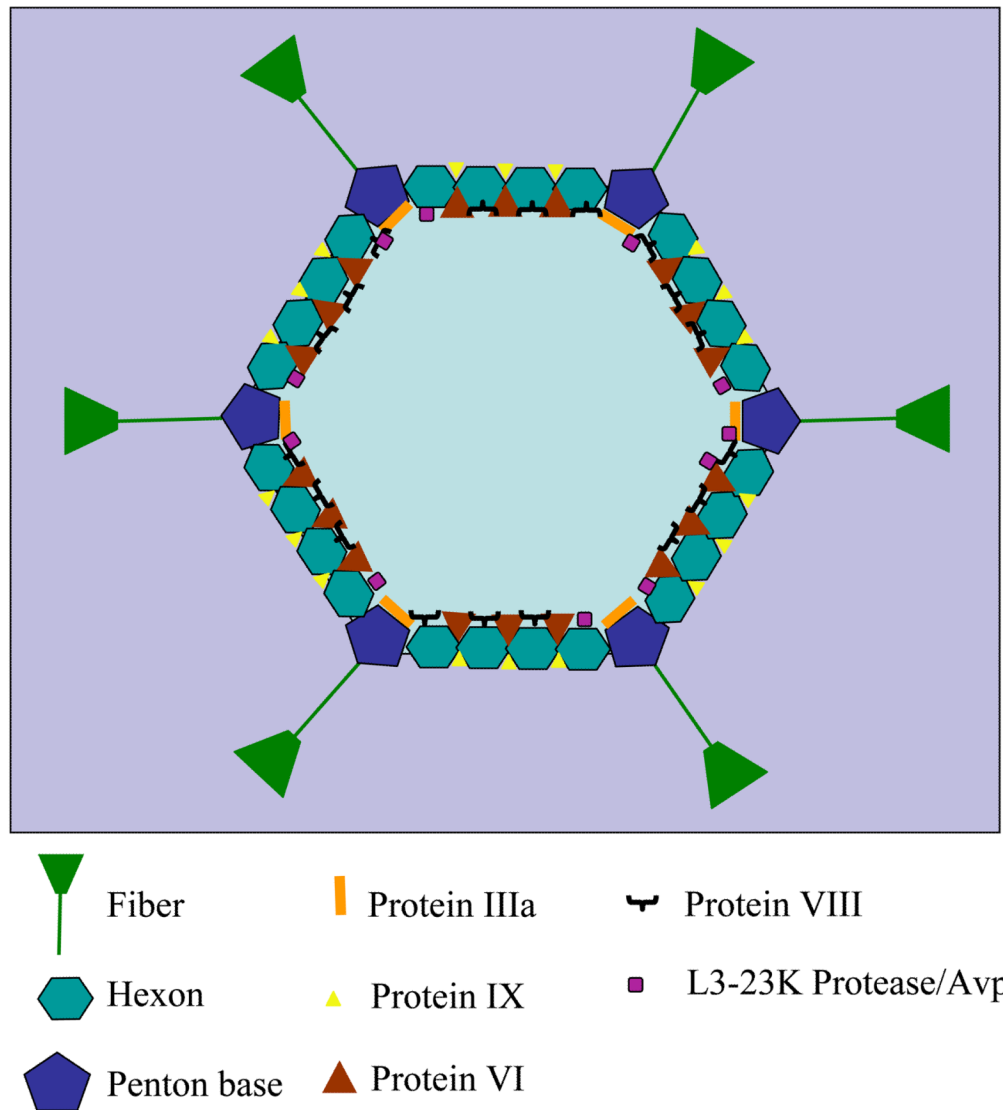
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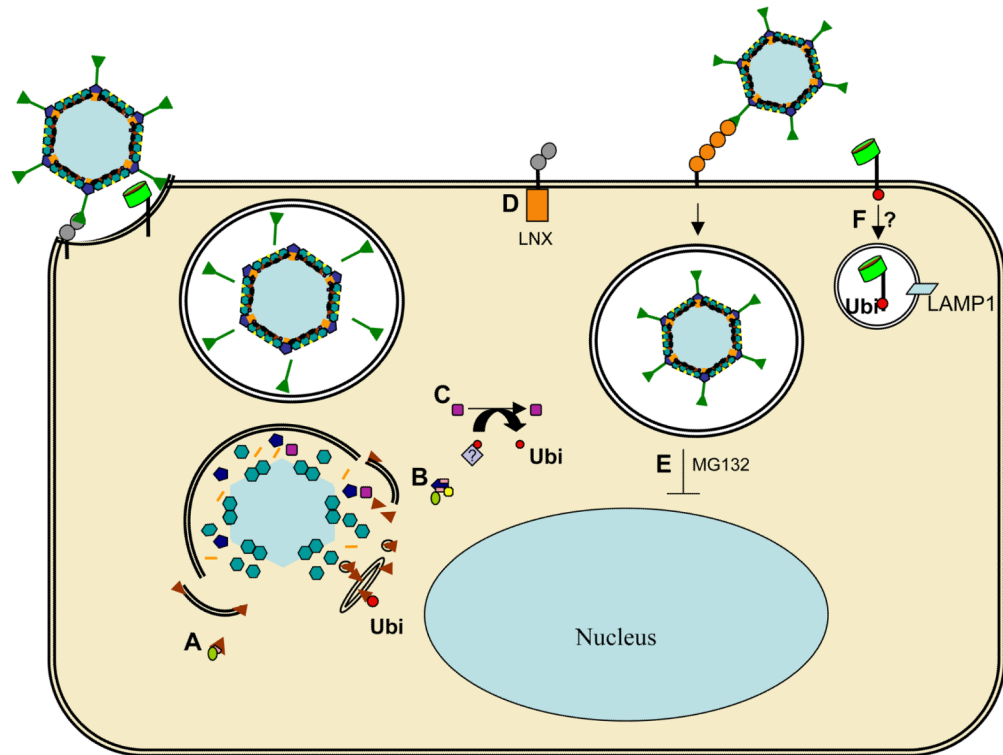
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**Figure 1. Adenovirus capsid structure**

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)



**Figure 2. Potential roles for ubiquitylation during adenovirus cell entry**

A) Protein VI contains a conserved PPxY motif, responsible for colocalization with Nedd4 family members of E3-ubiquitin ligases and *in vitro* pVI ubiquitylation. Mutation of the PPxY motif decreases specific infectivity at an entry step post endosomal escape. B) The penton base contains 2 conserved PPxY motifs, and Nedd4 E3-ubiquitin ligase family members bind penton base via the PPxY motifs. Additionally, penton base binds BAG3 in a PPxY-dependent manner. BAG3 knockdown decreases adenovirus specific infectivity. C) L3-23K protease/Avp activity is required during adenovirus cell entry. Avp possesses deubiquitylating activity, but it has not been determined if the deubiquitylating activity is required for adenovirus specific infectivity. D) CAR associates with the E3-ubiquitin ligase LNX. Not shown-MG132 treatment increases CAR surface expression and increases adenovirus infectivity. E) Treatment with MG132 decreases adenovirus infectivity for viruses that utilize the CD46 receptor. F) Upon adenovirus binding to  $\alpha_v\beta_3$  integrins, integrin ubiquitylation and trafficking to lysosomes (LAMP1) may occur.