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The Adenovirus Death Protein – A small membrane protein controls cell lysis and disease

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Abstract

Human adenoviruses (HAdVs) cause widespread acute and persistent infections. Infections are usually mild, and controlled by humoral and cell-based immunity. Reactivation of persistently infected immune cells can lead to a life-threatening disease in immuno-compromised individuals, especially children and transplant recipients. To date, no effective therapy or vaccine against HAdV disease is available to the public. HAdV species C type 2 and 5 are the best-studied of more than 100 HAdV types. They persist in infected cells and release their progeny by host-cell lysis to neighbouring cells and fluids, a process facilitated by the adenovirus death protein (ADP). ADP consists of about 100 amino acids and harbours a single membrane-spanning domain. It undergoes post-translational processing in ER and Golgi compartments, before localizing to the inner nuclear membrane. Here, we discuss the current knowledge on how ADP induces membrane rupture. Membrane rupture is essential for both progression of disease and efficacy of therapeutic viruses in clinical applications, in particular oncolytic therapy.

Keywords: Adenovirus death protein, apoptosis, cancer therapy, cell death, cell lysis, human adenovirus, membrane rupture, oncolytic viruses, virus egress, virus transmission

Abbreviations: aa, amino acid; ADP, adenovirus death protein; AMW, apparent molecular weight; *Atg*, autophagy-related genes; CCMV, chimpanzee cytomegalovirus; CMV, human cytomegalovirus; CR1, conserved region 1; COPI, coat protein complex type I; COPII, coat protein complex type II; CPE, cytopathic effect; ER, endoplasmic reticulum; HAdV, human adenovirus; MLP, major late promoter; NLS, nuclear localization sequence; ORF, open reading frame; PARP, poly (ADP-ribose) polymerase; RID, receptor internalization and degradation; RIPK1, receptor-interacting protein kinase 1; S1P, site 1 protease; S2P, site 2 protease; TGN, trans-Golgi network

Box: Key facts and hypotheses about ADP

- ADP transport between ER and Golgi apparatus is mediated by COPI- and COPII-coated vesicles.
- ADP undergoes N-glycosylation and it is then O-glycosylated at positions T_2 / S_4 / T_5 / T_9 / T_{10} by GalNAc-transferases T2 and T11, and proteolytically processed in the TGN
- The mature, cleaved ADP harbours a lysine/arginine based nuclear localization sequence in the cytosolic domain, and is translocated to the inner nuclear membrane by importin α/β.
- The palmitoylation at $C_{60}C_{61}$ enhances lipid-based sorting from ER / Golgi to the inner nuclear membrane and favours lipid raft association.
- ADP harbours a quadruplicate leucine zipper motif I_{71} to L_{99} in its cytosolic / nucleoplasmic C-terminal domain, which supports oligomerization. The preceding basic proline-rich region enhances DNA binding, destabilises the nuclear envelope, and promotes membrane rupture.

Human Adenoviruses and their disease

Human Adenoviruses (HAdVs) are wide-spread human pathogens (Lion, 2019; Lynch and Kajon, 2016). They comprise more than 100 types in the genus *Mastadenovirus* of the *Adenoviridae* family (Harrach, 2020; Harrach et al., 2019; Ismail et al., 2018). Their non-enveloped icosahedral capsid is about 90 nm in diameter, and contains emanating trimeric fiber proteins of variable length (Greber and Flatt, 2019; Nemerow et al., 2009). The capsid encloses a tightly packaged 34 to about 37 kbp DNA genome and viral proteins, the so called core (Benevento et al., 2014; Reddy et al., 2010). Based on hemagglutination assays, HAdVs were grouped into seven species A to G (Harrach et al., 2019). The HAdV types are heterogeneous regarding entry receptors, tissue tropism and associated disease (Arnberg, 2012; Khanal et al., 2018; Lynch and Kajon, 2016; Nemerow and Flint, 2019; Wolfrum and Greber, 2013).

One third of the known HAdV types is associated with human disease (Lynch and Kajon, 2016). While a subset of HAdV species D (HAdV-D) and E predominantly cause keratoconjunctivitis (Ismail et al., 2019), HAdV-A, B and C infections lead to urinary, respiratory and gastrointestinal disease, the latter can be also caused by HAdV-E and F (Lion, 2014; Lynch and Kajon, 2016). Antibody prevalence depends on the HAdV type and the geographic region, and can reach 95% (Mennechet et al., 2019; Sumida et al., 2005; Uusi-Kerttula et al., 2015; Vogels et al., 2003). Anti-HAdV antibodies are part of the adaptive immune system, and help to clear infection. In children and immuno-compromised patients, however, HAdV infection can lead to disseminated and potentially lethal disease (Lion, 2014). Especially transplant recipients and HIV-infected individuals are at risk to develop severe HAdV infections (Krilov, 2005; Lynch and Kajon, 2016), in part due to reactivation of virus production in persistently infected lymphocytes present in mucosal tissue of the digestive tract (Kosulin et al., 2016; Lion, 2014, 2019).

Upon infection, HAdV reprograms the cell and produces viral progeny (Figure 1). An indication of infection are morphological changes, such as rounding and the loss of cell adherence, so called cytopathic effect (CPE). CPE is in part based on the loss of cytoskeletal integrity, owing to the viral cysteine protease L3/p23 and E4 proteins modulating tight-junctions through interactions with PDZ domain-containing host proteins (Brown and Mangel, 2004; Chen et al., 1993; Glaunsinger et al., 2001; Greber, 1998; Staufenbiel et al., 1986). Virions assemble and mature in the nucleus (Mangel and San Martín, 2014). To complete a full productive viral replication cycle, the nuclear membrane and the plasma membrane of the infected cell rupture thereby releasing infectious viral progeny into the extracellular space. This lytic process gives rise to cell-free virions (Doronin et al., 2003; Tollefson et al., 1996a; Yakimovich et al., 2012). In cell culture, where a monolayer of cells is covered by aqueous medium, cell-free virions are transported asymmetrically from the lysed cell,

due to convective liquid movement (Yakimovich et al., 2012). This gives rise to comet-shaped infection foci, called plaques. Studying viral transmission in humans has been limited to end-point observations, such as biopsies or autopsy specimens, for example from immuno-compromised patients. Epithelial HAdV protein expression, gland epithelial necrosis and crypt apoptosis in histological sections of the gastrointestinal tract and virus shedding to the stool suggest that HAdV progeny is released from epithelial cells *in vivo* (Kaufman et al., 2002; Kosulin et al., 2016; Lion, 2019; Wong, 2015). Depolarization of infected intestinal epithelial cells, detachment and cell death have been observed akin to phenotypes in cell culture (Wong, 2015). The release of cell-free progeny may enhance intra- and inter-host pathogen transmission (Yakimovich et al., 2012, 2016).

HAdV transmission within solid organs is still poorly understood. Adenoviral hepatitis is characterized by patchy to extensive foci of infected cells, not restricted by the liver lobule and thus likely independent of cell-free virions in fluid transport through hepatic central veins or portal triads (Schaberg et al., 2017). Further analyses beyond confluent morphology and the necrotic core of these infection foci may eventually provide evidence for the transmission mode of HAdV in solid organs (Schaberg et al., 2017).

The currently best established animal model is the immuno-suppressed Syrian hamster, which is well susceptible to HAdV-C and to some extent to type B14 (Radke et al., 2016; Toth et al., 2017; Wold et al., 2019). HAdV-C6 primarily targets the liver when administered intravenously, but depending of the route of administration, HAdV-C replicates in most organs (Tollefson et al., 2017; Toth et al., 2008; Ying et al., 2009). Beside the Syrian hamster model, productive HAdV infection has been limited in other animal models, including pigs, rabbits and rats (reviewed in (Wold et al., 2019)). Foci of infected cells were found in liver sections of rats and the lungs of pigs upon intravenous inoculation, suggesting some level of productive HAdV replication (Haisma et al., 2008; Jogler et al., 2006). Acute infections are frequently accompanied by robust inflammatory responses, as shown in the airways or the conjunctiva of infected mice (Chintakuntlawar et al., 2007; Chodosh, 2006; Ginsberg et al., 1991; Kajon et al., 2003), reviewed in (Ismail et al., 2019). This reflects infection phenotypes in immuno-compromised patients, where HAdV infection foci are often infiltrated by immune cells (Orenstein and Dieterich, 2001; Schaberg et al., 2017; Wong, 2015), and disseminated disease causes morbidity (Lion, 2019; Lynch and Kajon, 2016).

E3 transcription unit

The proteins encoded by the early transcription unit E3 are dispensable for the replication of HAdV in cell culture but critically contribute to viral pathogenicity (Ginsberg et al., 1989; Kelly and Lewis,

1973). The E3 proteins are multifunctional and help the virus to evade host defence (Lichtenstein et al., 2004). The transcription unit E3 is composed of the E3a and E3b regions defined post-transcriptionally by poly(A) site selection. Both E3A and E3B pre-mRNAs are heavily spliced during processing (Donovan-Banfield et al., 2020; Scaria and Wold, 1994; Zhao et al., 2014) and yield five to nine proteins depending on the viral species. HAdV-C2 produces seven E3 proteins (see Figure 2A). The E3 transcription unit is the most divergent coding region, and exhibits the highest nucleotide diversity among HAdVs (Bair et al., 2017; Burgert and Blusch, 2000; Davison et al., 2003a, 2003b; Robinson et al., 2011, 2013). The 5' end of E3 encodes a 12.5K protein of unknown function in all species, apart from species F (Davison et al., 2003a). All E3 units have three conserved coding regions near their 3' end, receptor internalization and degradation α (RID-α), RID-β and 14.7K (Davison et al., 2003a), the products of which are involved in evasion of host cell death (Lichtenstein et al., 2004; Russell, 2009; Zeng and Carlin, 2019). RID-α and -β are transmembrane proteins, while 14.7K is cytosolic. The central E3 proteins harbour conserved region 1 (CR1) domains and are thus termed CR1 proteins (Deryckere and Burgert, 1996). The letters α to δ denote their position in the E3 region. The initial CR1 protein definition was later adopted for similar proteins without a CR1 domain, and additional CR1 proteins in the E3 region were mapped accordingly (Davison et al., 2003a). For example, E3A-19K is a CR1 protein, which lacks the CR1 domain but exhibits sufficient similarity to the other CR1 proteins.

The E3a CR1-β region of HAdV-C2 encodes for a 11.6 kDa protein, which facilitates host cytopathic effects and cell lysis at the end of the viral replication cycle (Tollefson et al., 1996a; Wold et al., 1984). This was demonstrated by the formation of smaller plaques formed by an E3a-11.6K-deleted HAdV-C5/C2 (dl712) (Tollefson et al., 1996a, 1996b). The deletion of other E3a or E3b genes did not affect the plaque size in cell cultures (Tollefson et al., 1996b), with the exception of E1B-19K-deleted mutants, which caused larger plaques (Chinnadurai, 1983; Gros et al., 2008). Accordingly, HAdV-C2 E3A-11.6K was named adenovirus death protein (ADP) (Tollefson et al., 1996a). The corresponding 10.5K ADP of HAdV-C5 is slightly smaller than the C2 protein (Wold et al., 1984). The C2 and C5 ADP exhibit 81% sequence identity, although the significance of the difference is unknown (for BLASTp analyses, see Table 1).

The CR1 domain shows strong similarity to the RL11 domain, an N-terminal protein motif first described in the human cytomegalovirus (CMV) multigene family RL11 (Chee et al., 1990). RL11 comprises 65-85 aa and a central conserved W…C…C motif, with nearby potential glycosylation sites. Homologous repetitive RL11/CR1 coding regions have been identified in chimpanzee CMV (CCMV) and HAdV, presumably resulting from genetic duplications, insertions and deletions (Davison et al., 2003a). With a few exceptions, all CMV, CCMV and HAdV proteins contain a

transmembrane domain and undergo N-terminal glycosylation. In HAdV, N- and O-glycosylation of CR1-γ (20.5K) of HAdV-B3 and B7, as well as an unusually large HAdV-D19 CR1-β of 49 kDa, have been described (Hawkins and Wold, 1995; Windheim and Burgert, 2002). Intriguingly, D19 CR1-β is proteolytically cleaved in the TGN, possibly by the same protease as ADP.

Presumably, all HAdV RL11/CR1 coding regions originate from a single ancestor and have evolved diverging functions correlating with different tissue tropisms of the viruses (Davison et al., 2003a; Lynch and Kajon, 2016). The CR1-α and CR1-δ of species HAdV-B, the CR1-α (6.7K) of HAdV-C, the UL5 and UL8 of CCMV, and the CMV UL5 and UL8 also contain a CR1 domain. The CR1-β region of the species C, ADP does not contain a RL11/CR1 domain (Davison et al., 2003a). HAdV-C 6.7K and the UL8 proteins of CCMV and CMV lack a cleavable signal peptide, akin to ADP. HAdV-C 6.7K and ADP are type III N-glycosylated transmembrane proteins (Lichtenstein et al., 2004). The 6.7K protein localizes primarily to the ER membrane and represses apoptosis by interacting with RID-β (Benedict et al., 2001). 6.7K also blocks intrinsic and extrinsic apoptosis and maintains Ca^{2+} homeostasis independent of other E3 proteins, and thus opposes the function of ADP (Moise et al., 2002). The CMV UL8 is a highly glycosylated late Ig-like protein that localizes to the cell surface (Pérez-Carmona et al., 2018). It down-modulates the inflammatory response to CMV infection, unlike ADP. Its Ig-like structure exhibits similarity to HAdV-D19 CR1-β. The HAdV-B1 E3 CR1-δ region shows expression kinetics and processing in ER / Golgi similar to ADP, but does not localize to the nucleus (Frietze et al., 2012). The deletion of its ortholog in HAdV-B3 did not affect progeny release from epithelial cells (Frietze et al., 2012). The uniqueness of the HAdV-C ADP protein is further supported by BLASTp queries of the NCBI database showing no similarities of the HAdV-C2 ADP domains M_1-D_{39}, M_{40}-C_{62} and L_{63}-D_{101} to other proteins than the HAdV-C CR1-β proteins, which are highly similar to each other (Figure 2B).

The cell lytic function of ADP

ADP is expressed from the E3 promoter at low levels early in infection (Tollefson et al., 1992), when viral proteins affecting cell cycle regulation, inhibition of apoptosis, immune evasion and viral DNA replication are expressed (Crisostomo et al., 2019; Pied and Wodrich, 2019) (see also Figure 2A). Later in the viral replication cycle, when progeny virions are assembled, ADP is expressed at high levels from the major late promoter (MLP) (Tollefson et al., 1992). This switch to high expression is facilitated by the L4-33K and L4-100K proteins (Farley et al., 2004). MLP-driven ADP expression was found to be promoted by L4-22K (Wu et al., 2012), a protein involved in late viral gene expression, viral DNA packaging and progeny virion production (Wu et al., 2012). L4-22K recruits the packaging proteins IVa2 and L1-52/55K. While the deletion of ADP from HAdV-C2/C5

has no effect on viral replication, its overexpression results in accelerated CPE, host-cell lysis and plaque formation (Doronin et al., 2000, 2003; Tollefson et al., 1996a, 1996b). Interestingly, early ADP mutants also lacked other E3 genes, such as 12.5K (deleted in VRX-007), 6.7K, 19K, 14.7K as well as RID-α and -β deleted in VRX-006 and VRX-007 (Doronin et al., 2003). Alternatively, modification of a splice acceptor site upstream of ADP in HAdV-C5 expressing a mutator DNA polymerase lead to increased ADP expression from the E3 transcription unit (Uil et al., 2011). Notably, ADP transcripts were generated at the expense of the upstream E3a genes 12.5K, 6.7K and 19K. The correlation between ADP expression levels and cell killing was clearly shown in epithelial A549 cells infected with wild type, ADP-deleted or over expressing HAdV-C5 viruses (Murali et al., 2014). These findings illustrate the importance of ADP in controlled induction of death of HAdV-C-infected host cells.

In addition, there are indications of tissue-specific cell lysis triggered by ADP. While HAdV-C-infected epithelial or fibroblast cell lines rapidly lyse, C2 and C5 infections of certain lymphoid cell lines proceed without cell killing and yield persisting infections (Flomenberg et al., 1996; Zhang et al., 2010). This phenotype resembles the infection of epithelial or fibroblast cells with species C in the presence of interferon, which suppresses the transcriptional activity of the E1A promoter (Prasad et al., 2020; Zheng et al., 2016). Persistence-prone lymphoid cell lines indeed express lower levels of ADP (Murali et al., 2014), consistent with the notion that the E3 transcription unit is under the control of E1A (Berk, 1986). However, overexpression of ADP in these lymphoid cell lines did not increase cell death, suggesting that additional factors are necessary to lyse these cells (Murali et al., 2014).

Direct non-lytic cell-to-cell transmission has been proposed for HAdV-F41, which causes gastroenteritis (Lynch and Kajon, 2016). This was based on the observation that neutralizing antibodies only partly reduced HAdV-F41 transmission in cell cultures (Siqueira-Silva et al., 2009). Remarkably, the genetic swap of HAdV-C5 ADP to the F41 E3 region resulted in a 10-50-fold increased release of cell-free HAdV-F41 progeny and a switch from slow growing, symmetrical plaques to fast growing comet-shaped plaques (Lu et al., 2013). Symmetrical plaques are indicative of non-lytic cell-to-cell transmission (Yakimovich et al., 2016). The data confirm the unique lytic function of HAdV-C ADP, and suggest that additional transmission mechanisms are exist in HAdV infection.

Maturation of ADP

ADP is a type III integral membrane protein with a single transmembrane signal-anchor sequence (M_{41}-L_{62}) of 22 amino acids (Scaria et al., 1992). It localizes to the endoplasmic reticulum (ER),

Golgi and inner nuclear membrane. With 101 amino acid (aa), HAdV-C2 ADP is slightly longer than the 93 aa HAdV-C5 ADP (Figures 2C, 2D). The transmembrane domain is essential for ADP function and cell lysis (Tollefson et al., 2003). The N-terminus (M_1-D_{40}) of ADP is located in the lumen and the C-terminus (K_{63}-D_{101}) protrudes to the cytosol (Scaria et al., 1992). In the ER, ADP is N-glycosylated co-translationally at N_{14} (Scaria et al., 1992), presumably by the oligosaccharyl transferase (Mohorko et al., 2011). The glycosylation at N_{14} supports ADP transport to the trans-Golgi network (TGN) (Scaria et al., 1992; Tollefson et al., 2003). Transport likely occurs via the coatamer protein complex type II (COPII) machinery (McCaughey and Stephens, 2018). N-linked glycosylation generally supports protein folding and protects from proteasomal degradation (Aebi et al., 2010; Helenius and Aebi, 2001, 2004). In the TGN, ADP is variably O-glycosylated at positions T_2 / S_4 / T_5 / T_9 / T_{10}, presumably by GalNAc-transferases T2 and T11 (Tollefson et al., 2003). The O-linked glycans are thought to protect ADP from proteasomal degradation, as inferred from other O-glycosylated proteins in mammalian cells (Van den Steen et al., 1998). They are, however, only transiently associated with ADP due to proteolytic processing of ADP in the TGN between T_{10} and M_{41} (Scaria et al., 1992). Neither the exact cleavage site nor the processing protease have been identified, and the fate of the cleaved N-terminal tail is unknown. Several proteases are suspected to be involved, and others can be ruled out. For example, a minimal furin protease recognition sequence (R-X-X-R) is missing in the lumenal domain of ADP (Molloy et al., 1992).

Potential processing sites, however, exist for site 1 protease (S1P) and site 2 protease (S2P). S1P localizes to the ER and Golgi complex, and cleaves lumenal proteins at small or hydrophobic amino acids preceded by R or K at position -4 (Nakagawa et al., 2017). S2P cleavage of lumenal proteins typically follows SP1 cleavage, and occurs in the TGN near the transmembrane domain preceded by R at position -4. In addition to R/K (-4), cleavage requires a proline residue at position 11 (Rawson, 2013; Ye et al., 2000). It is possible that S1P and/or S2P cleave ADP between T_{16}-|-A_{17} as well as at the interface between the N-terminal domain and the transmembrane domain. The former cleavage is supported by the observation that the deletion of residues D_{11}-L_{26} (dl736.1) and T_{18}-S_{22} (dl735), which removes the cleavage site and / or the proline at position 27, renders ADP unstable (Tollefson et al., 2003). A similar instability was observed upon deletion of S_4-D_{11} in the dl735 mutant. However, this deletion also removes the O-glycosylation sites, and thus may have other effects as well. The observation that the deletion of H_{29}-A_{45} (dl737) prevents the proteolytic cleavage of ADP suggests that the latter cleavage occurs N-terminal of the transmembrane domain, presumably by an unknown protease (Tollefson et al., 2003). The functions of the N-terminal proteolytic processing remain unknown, although N-terminal processing does not seem to affect cell lysis (Tollefson et al., 2003).

Furthermore, ADP undergoes cytosolic palmitoylation at C_{60} and C_{61} proximal to the transmembrane domain (Hausmann et al., 1998). The processed palmitoylated ADP is transported back to the inner membrane of the nuclear envelope, likely via coat protein complex type I (COPI) vesicles (Scaria et al., 1992). This exposes the C-terminus comprising K_{62}-D_{101} to the perinuclear space. The C-terminus harbours a proline-rich region with interspersed K/R residues at R_{64}-P_{74} and has high similarity to nuclear localization sequences (NLSs) (Tollefson et al., 2003). In support of this notion, ADP deleted in K_{63}-C_{94} failed to be transported to the nuclear membrane, and deletion of H_{81}-L_{88} lead to the formation of TGN-like vesicles containing ADP (Tollefson et al., 2003). These data support the notion that ADP uses a dedicated import pathway possibly requiring importin α/β binding to a cytosolic NLS, akin to other inner nuclear membrane proteins (Antonin et al., 2011; Bauer et al., 2015).

MAD2B - the only known ADP interaction partner

So far, only a single ADP-binding protein has been identified, the mitotic arrest deficient 2-like protein 2 (MAD2B), a homodimer of 211 amino acids each (Ying and Wold, 2003). Human MAD2B has 53% similarity with human MAD2, which is part of the mitotic spindle assembly checkpoint at kinetochores inhibiting the kinase CDC20, and thereby cell-cycle progression (Kops et al., 2005). MAD2B is a TCF4-binding protein (Hong et al., 2009). It localises to the nucleus and modulates epithelial-mesenchymal transition (Hong et al., 2009). MAD2B interacts with the DNA repair protein REV1 and the DNA polymerase ζ (Gupta et al., 2018; Hara et al., 2010; Murakumo et al., 2000, 2001; Zhang et al., 2007). Yeast-two hybrid, GST pull-down and co-immunoprecipitation experiments showed that MAD2B interacts with HAdV-C2 ADP (Ying and Wold, 2003). MAD2B binds to the cytosolic / nuclear C-terminus of ADP via $P_{69}P_{70}$ in the basic proline-rich region of ADP. Overexpression of MAD2B reduced HAdV-C2-induced lysis, suggesting that it neutralizes the lytic activity of ADP (Ying and Wold, 2003). It has not been established, however, how the interaction of MAD2B with ADP regulates lytic virus egress.

Many ways for cells to die

Advanced molecular tools yield an increasingly refined molecular classification of the cell death pathways, beyond morphological descriptions. Based hereon, the Nomenclature Committee on Cell Death 2018 acknowledges 12 distinct cell-death pathways (Galluzzi et al., 2018). One of them is classical apoptosis triggered by intrinsic or extrinsic signals. It depends on caspases and leads to the formation of apoptotic blebbing followed by cell shrinkage. This is distinct from necrosis, which occurs upon external stimuli, and induces membrane permeability as detected e.g. by

propidium iodide (PI) staining of the nucleus (Vitale et al., 1993). Necroptosis is a form of programmed necrosis, for example triggered by the activation of Toll-like receptors or virus infections (Lötzerich et al., 2018; Silke et al., 2015).

HAdV-C5 infection induces cell death, which involves membrane permeabilization indicated by PI-positive nuclei (Zou et al., 2004). This coincides with the release of progeny virions into the environment (Doronin et al., 2003; Yakimovich et al., 2012). The inhibition of apoptosis by chemical inhibitors or overexpression of anti-apoptotic proteins did not reduce death of the infected cells (Abou El Hassan et al., 2004; Zou et al., 2004). These findings argue for the induction of cell death processes other than apoptosis in HAdV infection. This would be akin to picornaviruses, where the viral protease 3C blocks apoptosis by cleaving the death domain of the host receptor-interacting protein kinase 1 (RIPK1) and thereby enables virus-controlled death (Lötzerich et al., 2018). In line with virus-controlled cell death, the mature ADP (M_{41}-D_{101}) has no similarity to other proteins in BLASTp searches of data banks, although it resembles the transmembrane domain of CR1-β in other species C HAdV and the lifeguard 1 protein in *Halyomorpha halys* [NCBI #XP_014271228.1].

Adenovirus-induced cell death

Regulation of cell death is an essential aspect of the replication cycle of many viruses, and its suppression supports oncogenic transformation. The release of cell-free progeny by lysis of host cells allows for long-range transmission of HAdV (Yakimovich et al., 2012). Yet, the virus evades innate immune responses and thereby maximizes the viral replication and progeny formation.

HAdVs have provided early insights into this double-edged process of death pathway regulation. HAdV-induced cell death exhibits features of apoptosis including cell shrinkage, membrane blebbing, activation of caspases 3 and 9, cleavage of poly (ADP-ribose) polymerase (PARP) and DNA degradation (Abou El Hassan et al., 2004; Braithwaite and Russell, 2001; Han et al., 1996; Ito et al., 2006; Jiang et al., 2007, 2011; Rao et al., 1992, 1996; Yun et al., 2005; Zou et al., 2004). An early activator of apoptosis is the immediate early viral trans-activator protein E1A, reviewed in (White, 2001). The pro-apoptotic activity of E1A maps to both retinoblastoma protein (Rb) and p300 binding sites of E1A and is completely independent of p53, which is in turn inactivated early in infection by the viral E1B-55K-E4orf6 complex. The pro-apoptotic signal triggered by E1A involves inappropriate cell-cycle progression, which includes the sequestration of Rb from E2F, and E2F mediated S-phase induction. Subsequent interaction of Bax and Bak triggers the release of cytochrome C from mitochondria and the activation of caspases 3 and 9. An additional viral feedback loop was identified using HAdV-C mutants lacking the viral E1B-19K, which is

functionally related to Bcl-2, reviewed in (Cuconati and White, 2002). Bcl-2 family proteins suppress mitochondrial permeability, and E1B-19K blocks E1A-mediated apoptosis by binding to Bak, which prevents the Bak-Bax oligomerization and ensuing cytochrome C release.

HAdV-induced cell death also exhibits autophagic features (Ito et al., 2006; Jiang et al., 2008, 2011; Klein et al., 2015; Piya et al., 2011; Rodriguez-Rocha et al., 2011). This pathway is induced by binding of E1B-19K to Beclin-1, also referred to as ATG6, one of the central regulators of autophagy (Cao and Klionsky, 2007; Piya et al., 2011). However, it remains controversial if autophagy is a cell-death pathway or rather establishes cellular homeostasis. In the context of infection, autophagy could represent a cellular defence mechanism. Another HAdV protein, E4orf4, was also demonstrated to induce host-cell death, reviewed in (Braithwaite and Russell, 2001; Kleinberger, 2019). More recently, insights into the molecular mechanism underlying E4orf4-mediated induction of cell death were reported (Dziengelewski et al., 2020). Ectopically expressed E4orf4 causes nuclear blebbing and rupture by binding to the polarity protein Par3 at the nuclear envelope. The E4orf4 motif necessary for Par3 binding is conserved across different HAdV species, and thus may execute pan-adenoviral transmission.

HAdV-C infection further leads to a progressive loss of cell adhesion (Rowe et al., 1958). This likely occurs through binding of the virion structural protein penton base to integrins, and the detachment of the anchored cells from the extracellular matrix (Wickham et al., 1993). In fact, viral capsomer proteins, including penton base and fiber, are released from the infected cell by a non-classical pathway prior to lysis (Trotman et al., 2003; Walters et al., 2002). In addition, HAdV alone or immune-complexed particles trigger pyroptosis of myeloid and epithelial cells, which involves inflammasome-dependent cytokine secretion and the activation of inflammatory caspases. This anti-viral response can lead to inflammatory tissue damage and is exacerbated by caspase-mediated cleavage of pore-forming gasdermins (Eichholz et al., 2016).

ADP-mediated cell death

Ever since ADP was discovered (Wold et al., 1984), the mechanisms by which it promotes HAdV egress and cell death have been debated. An early hypothesis has been that ADP initiates membrane permeabilization. Indeed, ADP has similarity to viroporins, hydrophobic oligomerizing membrane pore-forming proteins (Gonzalez and Carrasco, 2003; Hausmann et al., 1998; Nieva et al., 2012). We hypothesize the presence of a quadruplicate leucine zipper motif I_{71} to L_{99} in the cytosolic / nucleoplasmic ADP C-terminal domain, which is indicated by orange triangles in Figure 2C. In this region, L, M, V and I are interspersed with 6 - 7 amino acids, typical of a quadruplicate leucine zipper motif (Bornberg-Bauer et al., 1998; Hakoshima, 2001). Leucine zippers are known

to induce dimerization or oligomerization of proteins (Hakoshima, 2001). Moreover, the proposed domain of ADP is preceded by basic amino acids, which may aid DNA binding (Hakoshima, 2001). Such ADP-DNA binding might potentially facilitate chromatin sequestration from HAdV replication centres and lead to nuclear destabilization.

Nuclear envelope destabilization by ADP is worth considering as a mechanism for rupturing the nuclear envelope and releasing progeny virus particles from the nucleoplasm to the cytosol, and eventually from the infected cell (Puntener et al., 2011; Tollefson et al., 1996a). We speculate that ADP enhances nuclear envelope instability and potentiates biophysical cues from HAdV replication and progeny assembly in the nucleus, including nuclear expansion by an increase in physical pressure (Andriasyan et al., 2019). It is conceivable that the palmitoylation at $C_{60}C_{61}$ enhances lipid-based sorting of the cleaved form of ADP to the inner nuclear membrane (Blaskovic et al., 2013; Hausmann et al., 1998; Scaria et al., 1992; Tollefson et al., 2003). This would be in line with the notion that the transmembrane domain of ADP is shorter than the average length of mammalian membrane-spanning domains, and appears to be well adapted to the cholesterol-low membranes of the ER and the nuclear envelope (Munro, 1995; Singh and Mittal, 2016). Interestingly, ADP may counteract the apoptosis-suppressing function of E1B-19K (Hausmann et al., 1998), and thereby give rise to larger sized plaques and increased anti-tumour efficacy, as has been observed in E1B-19K-deleted HAdV (Chinnadurai, 1983; Gros et al., 2008; Liu et al., 2004). Notably, E1B-19K is acylated (palmitoylated and myristoylated) and localizes to the ER and the nuclear envelope (McGlade et al., 1987; White et al., 1984). The localization of E1B-19K and ADP in the nuclear membrane supports the notion that the plaque-size defect of dl327 (lacking ADP and other E3 coding regions) can be compensated by mutations in E1B-19K and E1B-55K (Subramanian et al., 2006; Thimmappaya et al., 1982). Whether ADP synergizes with E1B-19K in inhibiting Bak/Bax remains to be investigated. Likewise, it is unknown if ADP potentiates the pro-apoptotic effects of other early viral genes, such as E4orf4 (Gingras et al., 2002; Kleinberger, 2019; Livne et al., 2001; Marcellus et al., 1998; Miron et al., 2009; Mui et al., 2013; Robert et al., 2002).

ADP in cancer therapy

The selective killing of cancer tissues and their metastases is the ultimate goal of cancer therapy. Oncolytic viruses selectively replicate in neoplastic tissues and hold significant promise for the treatment of cancer (Russell and Peng, 2017; Yamamoto and Curiel, 2010). One of the first reported cases of cancer remission by a virus was from a woman receiving a live-attenuated rabies vaccine (DePace, 1912). Subsequent clinical observations showed that sometimes virus infections correlated with cancer regression (Bluming and Ziegler, 1971; Hoster et al., 1949;

Zygiert, 1971). Clinical trials with a range of human and animal viruses then showed that most viruses were ineffective against the cancer and were eliminated by the immune system of the host (Huebner et al., 1956; Russell et al., 2012). This indicated that direct and effective cancer-cell killing was required to fight cancer in immunocompetent patients. In turn, the lytic nature of HAdV infection was discovered in 1953 (Hilleman and Werner, 1954; Rowe et al., 1953) and this spurred intense interest to develop oncolytic therapy based on a variety of HAdV types (Huebner et al., 1956; Yamamoto and Curiel, 2010).

The viral proteins involved in host-cell death remain incompletely resolved, although many of them have been identified. Oncolytic HAdV based vectors were used to investigate the role of HAdV proteins in tumour-cell killing, reviewed in (Farrera-Sal et al., 2020; Gros and Guedan, 2010; Stepanenko and Chekhonin, 2018; Toth et al., 2010). Regarding ADP (Tollefson et al., 1996a), ADP-overexpressing HAdV mutants were designed for oncolytic therapy. HAdV-C5 mutants KD1 and KD3 lacked all E3 genes apart from 12.5K and ADP (Doronin et al., 2000). To enhance tumour-cell selectivity, they also harboured two E1A mutations impairing E1A binding to Rb and p300 (Howe et al., 1990). Tumour-cell killing specificity was improved by replacing the E4 promoter by the promoter for surfactant protein B (Doronin et al., 2001). Both oncolytic viruses showed increased efficacy in xenograft models compared to wild type HAdV-C5 (Doronin et al., 2001). Meanwhile, another ADP-overexpressing HAdV-C5-based oncolytic virus was generated in a comparable approach (Ramachandra et al., 2001). The E3 region with the exception of 12.5K of 01/PEME was replaced by a MLP-ADP overexpression cassette. 01/PEME is further mutated in the same N-terminal E1A region as KD1 and KD3. Accordingly, also this ADP-overexpressing oncolytic virus demonstrated increased selectivity and potency in tumour-cell killing *in vitro* and *in vivo* (Ramachandra et al., 2001). Intriguingly, the re-introduction of an ADP CMV overexpression cassette into E1B-55K/E3-deleted HAdV-C5 vector YKL-1 led to enhanced tumour-cell killing *in vitro* and *in vivo*, and increased the size of viral plaques, yet apparently had no effects on normal skin fibroblasts (Kim et al., 2003; Lee et al., 2000; Yun et al., 2005). Also the ADP-overexpressing Ad5-yCD/*mut*TK_{SR39}rep-ADP outperformed its parental E1B-55K- and ADP-deleted virus regarding anti-tumour effects and specificity *in vitro* and in xenograft models (Barton et al., 2006; Freytag et al., 1998). Taken together, ADP overexpression can potentiate the efficacy of oncolytic viruses.

However, severe host immune responses and tumour access are an unresolved issue with oncolytic AdV in gene therapy (Khare et al., 2011; Marelli et al., 2018). It is clear that safety issues and enhanced tumour killing by AdV vectors will implement at least three strategies - arming, targeting, and shielding. This will involve better control of local inflammation and targeted cell killing, perhaps by employing ADP (Breitbach et al., 2007; Cervera-Carrascon et al., 2019; Gros

and Guedan, 2010; Machiels et al., 2019; Nemerow and Flint, 2019; Rosewell Shaw and Suzuki, 2016).

Future questions in ADP research

Pathogen-associated molecular patterns of viruses invariably trigger the onset of cell-death processes. This is detrimental to the dissemination of the virus. Viruses have evolved a range of countermeasures, including the abrogation of cell-controlled death processes and the execution of virus-controlled death processes. This allows viruses to take control of the timing and the molecular pathway of cell death. Viruses thereby control how they disseminate in an infected organism and between organisms. Given the considerable cell-to-cell variability in HAdV infection phenotypes (Suomalainen et al., 2020), the next frontier is to analyse the viral mechanisms of cell killing at the singl- cell level. This will involve improved image-based approaches (Pied and Wodrich, 2019; Witte et al., 2018). Remarkably, microscopic fluorescence-based methods revealed that only a minority of HAdV-C2-infected cells lyse and give rise to a productive infection, a plaque (Yakimovich et al., 2012). Deep learning-based imaging already allows predictions of infection outcome, for example lytic / non-lytic, and thereby allows studying features of lytic or lysogenic cells (Andriasyan et al., 2019). Additionally, genetic manipulations have been significantly eased by the implementation of the CRISPR/Cas system (Chen et al., 2020). In combination with high-throughput, genome-wide screening platforms (Lian et al., 2019), genotype-phenotype relations can today be addressed in an unbiased manner. In conclusion, the ADP case should be re-opened and the role of ADP in host-cell lysis addressed using state-of-the-art techniques. ADP can be studied both in the context of infection, upon expression of recombinant protein alone or in combination with viral proteins, such as E1B-19K. Promising future approaches include the generation of imaging-compatible tagged ADP mutants, as well as studies in artificial lipid bilayers. Gaining a deeper understanding of how the different HAdV species induce cell death and how this affects virus transmission between cells will likely reveal new therapeutic targets for the treatment of HAdV infection.

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Figures and Tables

Figure 1: Overview of the HAdV-C replication cycle. In a primary infection of epithelial cells, incoming virions bind to CAR receptors and, while bound to CAR, move on the plasma membrane by retrograde actin flow (Bergelson et al., 1997; Burckhardt et al., 2011; Kotha et al., 2015; Lütschg et al., 2011). Engagement of incoming virions with integrin receptors confines virions to small areas of submicron size on the plasma membrane (Nagel et al., 2003; Wickham et al., 1993). This triggers cell signalling (Wolfrum and Greber, 2013), the shedding of the fibers viral proteins, and the exposure of limited amounts of the membrane lytic virion protein VI (Greber, 2016; Nakano et al., 2000). Protein VI leads to the formation of small lesions in the plasma membrane, which triggers repair processes by lysosomal secretion (Burckhardt et al., 2011; Luisoni et al., 2015; Wodrich et al., 2010). This is rapidly followed by virion endocytosis (Greber et al., 1993; Meier et al., 2002). HAdV-C particles escape from an early endosome by a pH-independent process involving the membrane lytic protein VI and ceramide lipids (Luisoni et al., 2015, 2016; Suomalainen et al., 2013; Wiethoff and Nemerow, 2015). Cytosolic particles are transported bidirectionally on microtubules by kinesin and dynein motors, detach from microtubules proximal to the nucleus, and dock at the nuclear pore complex (NPC) (Gazzola et al., 2009; Trotman et al., 2001). NPC-docked virions disassemble and release their DNA genome (vDNA) into the nucleus upon priming by the E3 ubiquitin ligase Mib1 and capsid disruption by kinesin-1 (Bauer et al., 2019; Strunze et al., 2011). Within the nucleus, the viral genome is transcribed by the cellular RNA polymerase 2 which gives rise to mRNAs and eventually proteins, such as the immediate early E1A trans-activator, which boosts all the sub-viral promoters, and drives the cell into the S-phase where efficient viral DNA replication occurs (Berk, 2005). The expression of the early E2, E3 and E4 transcription units mediate immune escape (Atasheva et al., 2019; Kleinberger, 2015; Sohn and Hearing, 2019). The E3-19K protein initiates the unfolded protein response by selective activation of the IRE1 sensor in the endoplasmic reticulum (ER), and enhances both lytic and persistent infection (Prasad et al., 2020). Viral replication compartments in the nucleus are formed several hours after the delivery of viral DNA in the nucleus depending on the cell type, and cause severe morphological changes in the nucleus (Charman et al., 2019; Hidalgo and Gonzalez, 2019; Lynch et al., 2019; Puvion-Dutilleul et al., 1998). During late stages of HAdV-C replication, predominantly transcription units L1-5 are expressed and give rise to structural proteins and progeny virions (Martín-González et al., 2019). Virion assembly gives rise to large clusters of particles and capsomers HAdV assembly involves packaging of the viral genome and maturation of precursor proteins by the HAdV protease (Greber, 1998; Mangel and San Martín, 2014). Mature HAdV progeny is released upon cell lysis, where the nuclear envelope and the plasma membrane rupture and give rise to cell-free virions, and secondary infections (Murali et al., 2014). Much of the information listed here has been derived from virus imaging. For additional details, the reader is referred to recent review articles (Greber and Flatt, 2019; Nemerow and Flint, 2019; Pied and Wodrich, 2019; Wang et al., 2018; Witte et al., 2018).

Figure 2: The E3 transcription unit of HAdV-C and the processing of E3-11.6K / ADP. A The E3a transcription unit of HAdV-C2 (blue) encodes for the 11.6K protein ADP from the coding region CR1-β. E3b open reading frames (ORFs) are shown in green. At early infection stages, ADP is expressed at low levels

from the E3 promoter, represented as a grey arrow, located upstream of the E3 transcription unit. During late infection, ADP mRNA is transcribed from the major late promoter (MLP, transcription initiation indicated by a grey arrow). Donor and acceptor sites for mRNA splicing are indicated as grey empty and full triangles, respectively. Polyadenylation sites are represented by AAA symbols. **B** Comparison between the hydrophobicity profiles of ADP proteins encoded by the five known HAdV-C members. Hydrophilic and hydrophobic residues are depicted in blue and red, respectively. **C** ADP is post-translationally processed and harbours multiple confirmed and suspected domains. The N-terminal domain (purple) is O- and N-glycosylated, as indicated by pink and red symbols, respectively. O-glycosylation* indicates that modified residues have not been mapped individually. The area with the suspected cleavage sites C-terminal of the glycosylated domain is shown in green. The transmembrane domain is shown in red, with two nearby palmitoyl groups at the terminal Cys (C) residues shown in green. A basic proline-rich region is adjacent to the transmembrane domain, as indicated by yellow letters representing the corresponding amino acids (aa). The cytosolic domain further harbours hydrophobic amino acids (L and I) arranged in a leucine zipper-like pattern, indicated with orange triangles. **D** ADP, which is membrane associated, is translocated from the endoplasmic reticulum (ER) via the trans-Golgi network (TGN) to the nucleus. Zoomed in graphics indicate the processing step occurring in the respective organelles. On the right side, schematics of differentially processed ADP with the corresponding organellar location. The apparent molecular weight (AMW) of each ADP variant is derived from analyses of cell lysates by SDS-PAGE. O-glycosylation* indicates that modified residues have not been mapped individually.

Table 1: CR1-β / ADP sequences in HAdV-C obtained from the NCBI protein database (ncbi.nlm.nih.gov/protein/).

HAdV spe-cies	HAdV type	NCBI Reference Sequence	Name	aa Sequence	aa Length	Weight [kDa]
C	1	AAQ10560.1	10.7 kDa protein	MVDTVNSYNTATGLTSTQDMPQVSTFVNN WANLGMWWFSIALMFVCLIIMWLSCCLKRK RARPPIYKPIIVLNPNNDGIHRLDGLNTCSFS FAV	94	10.7
C	2	AAA92222.1	11.6 kD protein	MTGSTIAPTTDYRNTTATGLTSALNLPQVHA FVNDWASLDMWWFSIALMFVCLIIMWLICCL KRRRARPPIYRPIIVLNPHNEKIHRLDGLKPC SLLLQYD	101	11.7
C	5	AP_000221.2	10.5 kD protein	MTNTTNAAAATGLTSTTNTPQVSAFVNNWD NLGMWWFSIALMFVCLIIMWLICCLKRKRAR PPIYSPIIVLHPNNDGIHRLDGLKHMFFSLTV	93	10.5
C	6	ACN88121.1	ADP glycoprote in CR1-beta0	MTGSTIAPTTDYRNTTATGLKSALNLPQVHA FVNDWASLGMWWFSIALMFVCLIIMWLICCL KRRRARPPIYRPIIVLNPHNEKIHRLDGLKPC SLLLQYD	101	11.6
C	57	ADM46163.1	CR1 beta 11.6 kDa protein	MTGSTIAPTTDYRNTTATGLKSALNLPQVHA FVNDWASLGMWWFSIALMFVCLIIMWLICCL KRRRARPPIYRPIIVLNPHNEKIHRLDGLKPC SLLLQYD	101	11.6

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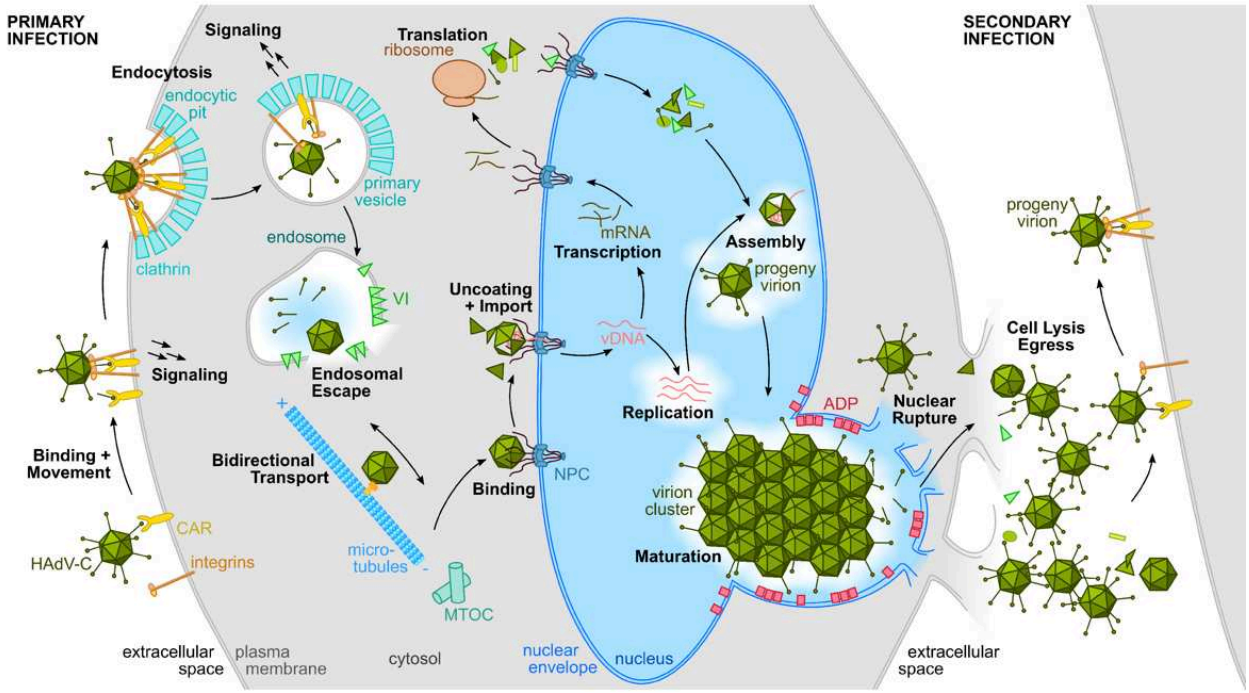
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Signaling
Translation
ribosome
SECONDARY INFECTION
Endocytosis
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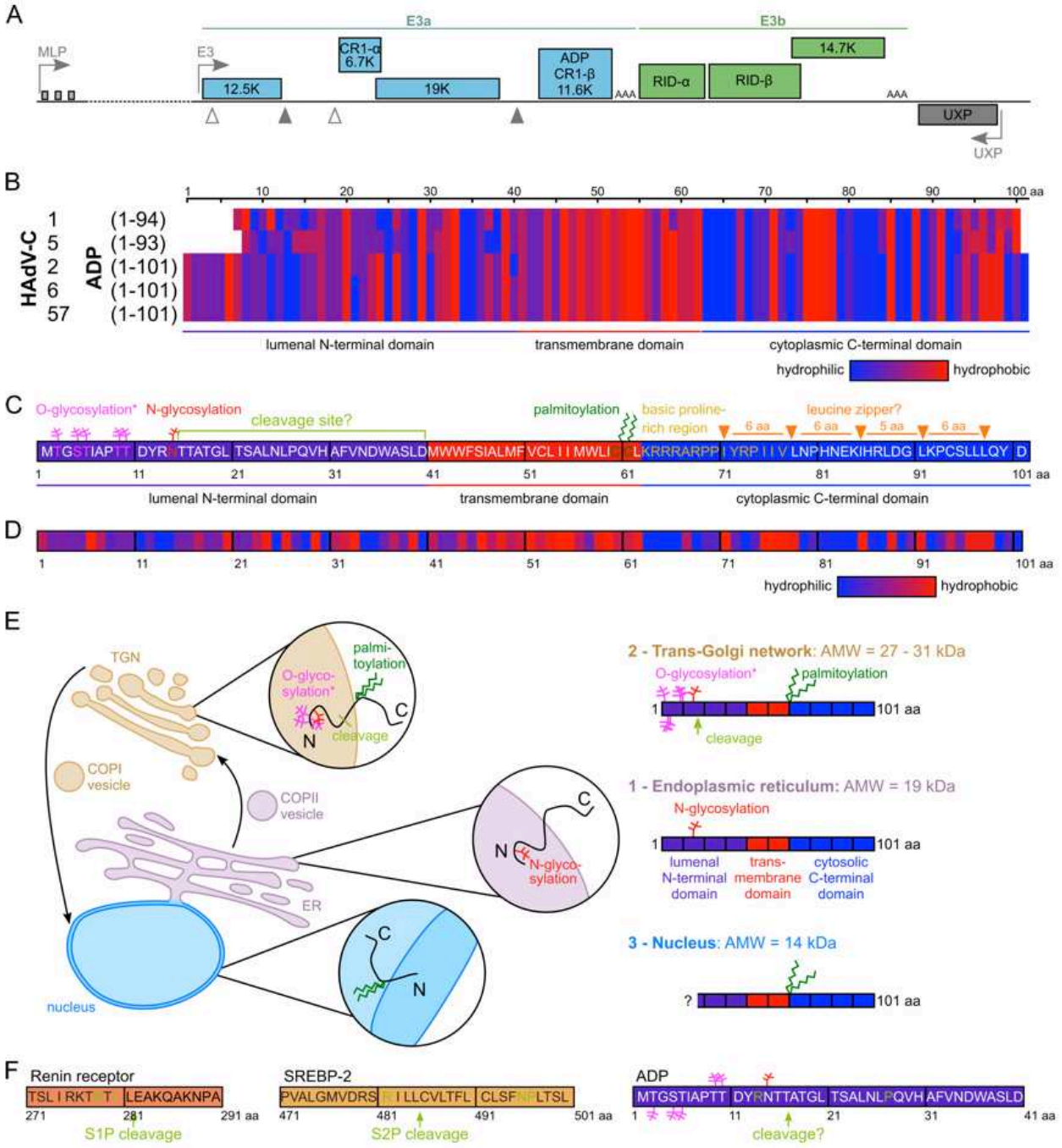
primary vesicle
clathrin
endosome
VI
Endosomal Escape
mRNA
Transcription
Assembly
progeny virion
progeny virion
Uncoating + Import
vDNA
Signaling
Replication
ADP
Nuclear Rupture
Cell Lysis Egress
Binding + Movement
Bidirectional Transport
Binding
NPC
virion cluster
Maturation
CAR
HAdV-C
integrins
micro-tubules
MTOC
extracellular space
plasma membrane
cytosol
nuclear envelope
nucleus
extracellular space

F2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E3a
E3b
MLP
E3
CR1-α 6.7K
12.5K
19K
ADP CR1-β 11.6K
RID-α
RID-β
14.7K
AAA
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lumenal N-terminal domain
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cytoplasmic C-terminal domain
hydrophilic
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C
O-glycosylation*
N-glycosylation
cleavage site?
palmitoylation
basic proline-rich region
leucine zipper?
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lumenal N-terminal domain
transmembrane domain
cytoplasmic C-terminal domain
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hydrophilic
hydrophobic
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TGN
palmi-toylation
O-glyco-sylation*
cleavage
COPI vesicle
COPII vesicle
ER
N-glyco-sylation
nucleus
2 - Trans-Golgi network: AMW = 27 - 31 kDa
O-glycosylation*
palmitoylation
cleavage
1 - Endoplasmic reticulum: AMW = 19 kDa
N-glycosylation
lumenal N-terminal domain
trans-membrane domain
cytosolic C-terminal domain
3 - Nucleus: AMW = 14 kDa
F
Renin receptor
SREBP-2
ADP
S1P cleavage
S2P cleavage
cleavage?

Tab 1

Supplementary Table 1: Published CR1-β / ADP sequences in HAdV-C obtained from the NCBI protein database (ncbi.nlm.nih.gov/protein/).

HAdV spe-cies	HAdV type	NCBI Reference Sequence	Name	aa Sequence	aa Length	Weight [kDa]
C	1	AAQ10560.1	10.7 kDa protein	MVDTVNSYNTATGLTSTQDMPQVSTFVNN WANLGMWWFSIALMFVCLIIMWLSCCLKRK RARPPIYKPIIVLNPNNDGIHRLDGLNTCSFS FAV	94	10.7
C	2	AAA92222.1	11.6 kD protein	MTGSTIAPTTDYRNTTATGLTSALNLPQVHA FVNDWASLDMWWFSIALMFVCLIIMWLICCL KRRRARPPIYRPIIVLNPHNEKIHRLDGLKPC SLLLQYD	101	11.7
C	5	AP_000221.2	10.5 kD protein	MTNTTNAAAATGLTSTTNTPQVSAFVNNWD NLGMWWFSIALMFVCLIIMWLICCLKRKRAR PPIYSPIIVLHPNNDGIHRLDGLKHMFFSLTV	93	10.5
C	6	ACN88121.1	ADP glycoprote in CR1-beta0	MTGSTIAPTTDYRNTTATGLKSALNLPQVHA FVNDWASLGMWWFSIALMFVCLIIMWLICCL KRRRARPPIYRPIIVLNPHNEKIHRLDGLKPC SLLLQYD	101	11.6
C	57	ADM46163.1	CR1 beta 11.6 kDa protein	MTGSTIAPTTDYRNTTATGLKSALNLPQVHA FVNDWASLGMWWFSIALMFVCLIIMWLICCL KRRRARPPIYRPIIVLNPHNEKIHRLDGLKPC SLLLQYD	101	11.6