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REVIEW ARTICLE

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

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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 immunocompromised individuals, especially children and transplant recipients. To date, no effective therapy or vaccine against HAdV disease is available to the public. HAdV-C2 and C5 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 endoplasmic reticulum 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

Human adenoviruses and their disease

Human adenoviruses (HAdVs) are widespread pathogens of the *Adenoviridae* family [1,2]. They comprise more than 100 types in the genus *Mastadenovirus* [3–5]. Their nonenveloped icosahedral capsid is about 90 nm in diameter and contains emanating trimeric fibre proteins of variable length [6,7]. The capsid encloses a tightly packaged 34 to about 37 kbp DNA genome and viral proteins, the so-called core [8,9]. Based on hemagglutination assays, HAdVs were grouped into seven species A to G [4]. The HAdV types are heterogeneous regarding entry receptors, tissue tropism and associated disease [2,10–13].

One-third of the known HAdV types is associated with human disease [2]. While a subset of HAdV species D (HAdV-D) and E predominantly cause keratoconjunctivitis [14], HAdV-A, B and C infections lead to urinary, respiratory and gastrointestinal disease, the latter also caused by HAdV-E and F [2,15]. Antibody prevalence depends on the HAdV type and the geographic region, and can reach 95% [16–19]. Anti-HAdV antibodies help to clear infection. In children and immunocompromised patients, however, HAdV infection can lead to disseminated and potentially lethal disease [15]. Especially, transplant recipients and

Abbreviations
aa, amino acid; ADP, adenovirus death protein; CCMV, chimpanzee cytomegalovirus; CMV, human cytomegalovirus; COPI, coat protein complex type I; COPII, coat protein complex type II; CPE, cytopathic effect; CR1, conserved region 1; ER, endoplasmic reticulum; HAdV, human adenovirus; MLP, major late promoter; NLS, nuclear localization sequence; RID, receptor internalization and degradation; S1P, site-1 protease; S2P, site-2 protease; TGN, trans-Golgi network.

Key facts and hypotheses about ADP

- The transport of ADP between endoplasmic reticulum (ER) and Golgi apparatus is mediated by COPI- and COPII-coated vesicles.
- ADP undergoes N-glycosylation and is 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, destabilizes the nuclear envelope and promotes membrane rupture.

HIV-infected individuals are at risk to develop severe HAdV infections [2,20], in part due to reactivation of virus production in persistently infected lymphocytes of mucosal tissue in the digestive tract [1,15,21].

Upon infection, HAdV reprograms the cell and produces viral progeny (Fig. 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 [22–26]. Virions assemble and mature in the nucleus [27]. 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 [28–30]. 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 [30]. This gives rise to comet-shaped infection foci. Viral transmission studies in humans have been limited to end-point observations from biopsies, or autopsy specimens in immunocompromised patients for example. Epithelial HAdV protein expression, gland epithelial necrosis and crypt apoptosis in the gastrointestinal tract together with virus shedding to the stool suggest that HAdV progeny is disseminated from epithelial cells *in vivo* [1,21,31,32]. Depolarization of infected intestinal epithelial cells, detachment and cell death have been observed akin to phenotypes in cell culture [32]. The release of cell-free progeny may enhance intra- and interhost pathogen transmission [30,33].

Human adenoviruses transmission within organs is still poorly understood, although adenoviral hepatitis gives rise to patchy or extended infection foci, not restricted by the liver lobule and thus likely independent of cell-free virions transported in hepatic central veins or portal triads [34]. Further analyses are required to provide more mechanistic insight into the transmission mode of HAdV in organs [34].

The currently best-established animal model for HAdV is the immunosuppressed Syrian hamster, which is well susceptible to HAdV-C and to some extent to type B14 [35–37]. HAdV-C6 primarily targets the liver when administered intravenously, but depending on the route of administration, HAdV-C replicates in most organs [38–40]. Beside the Syrian hamster model, productive HAdV infection has been tested in pigs, rabbits and rats, albeit found to be limited (reviewed in Wold *et al.* [37]. For example, 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 [41,42]. Acute infections are frequently accompanied by robust inflammatory responses, as shown in the airways or the conjunctiva of infected mice [43–46], reviewed in Ismail *et al.* [14]. This resembles infection phenotypes in immunocompromised patients, where HAdV infection foci are often infiltrated by immune cells [32,34,47], and disseminated disease causes morbidity [1,2].

E3 transcription unit

The proteins encoded in the early transcription unit E3 are dispensable for the replication of HAdV in cell culture but critically contribute to viral pathogenicity [48,49]. The E3 proteins are multifunctional and help the virus to evade host defence [50]. 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 [51–53] and yield five to nine proteins depending on the viral species. HAdV-C2 produces seven E3 proteins (Fig. 2A). The E3 transcription unit is the most divergent coding region and exhibits the highest nucleotide diversity among HAdVs [54–59]. The 5′ end of E3 encodes a 12.5K protein of unknown function in all

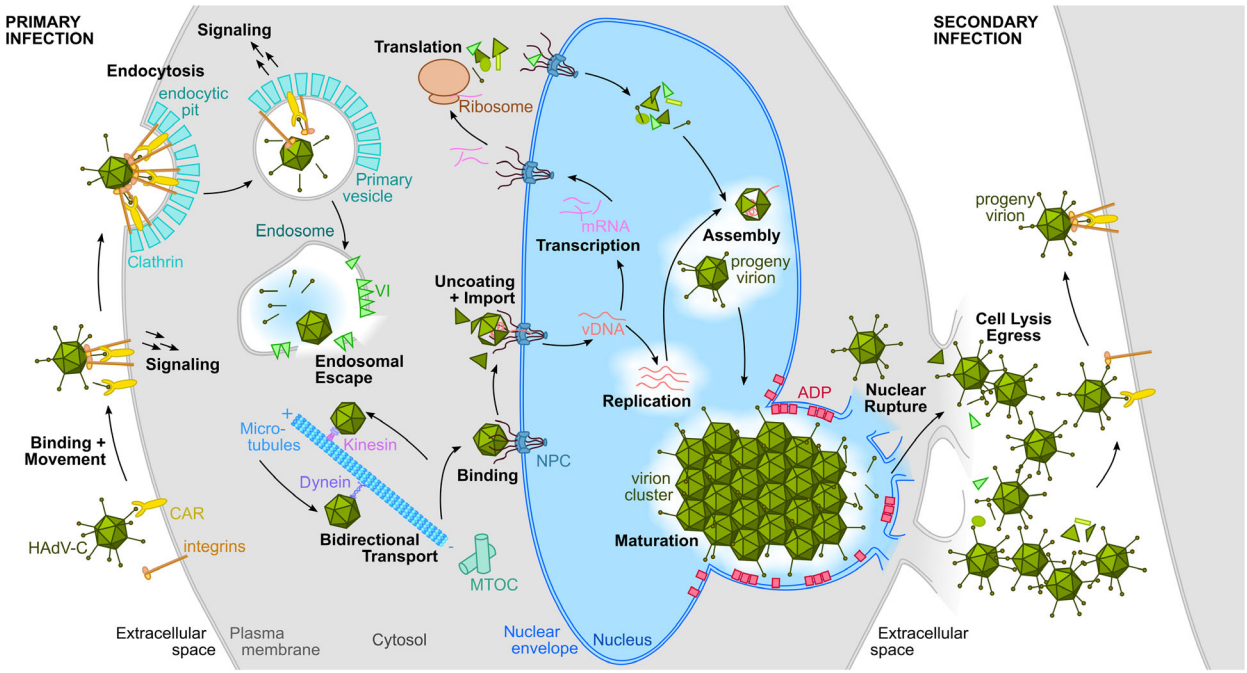

Fig. 1. Overview of the HAdV-C replication cycle. In a primary infection of epithelial cells, incoming virions bind to CAR receptors and, while bound to CAR, move on the plasma membrane by retrograde actin flow [191–194]. Engagement of incoming virions with integrin receptors confines virions to small areas of submicron size on the plasma membrane [136,195]. This triggers cell signalling [13], the shedding of the fibre viral proteins and the exposure of limited amounts of the membrane lytic virion protein VI [196,197]. Protein VI leads to the formation of small lesions in the plasma membrane, which triggers repair processes by lysosomal secretion [192,198,199]. This is rapidly followed by virion endocytosis [200,201]. HAdV-C particles escape from an early endosome by a pH-independent process involving the membrane lytic protein VI and ceramide lipids [198,202–204]. 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) [205,206]. 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 [207,208]. 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 transactivator, which boosts all the subviral promoters, and drives the cell into the S-phase where efficient viral DNA replication occurs [209]. The expression of the early E2, E3 and E4 transcription units mediates immune escape [210–212]. The E3-19K protein initiates the unfolded protein response by selective activation of the IRE1 sensor in the ER and enhances both lytic and persistent infection [84]. 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 [213–216]. During late stages of HAdV-C replication, predominantly transcription units L1-5 are expressed and give rise to structural proteins and progeny virions [217]. Virion assembly gives rise to large clusters of particles. Capsomer assembly involves packaging of the viral genome and maturation of precursor proteins by the HAdV protease [25,27]. Mature HAdV progeny is released upon cell lysis, where the nuclear envelope and the plasma membrane rupture and give rise to secondary infections [81]. Much of the information listed here has been derived from virus imaging. For additional details, the reader is referred to recent review articles [6,12,76,188,218].

species, apart from species F [56]. All E3 units have three conserved coding regions near their 3' end, receptor internalization and degradation α (RID-α), RID-β and 14.7K [56], the products of which are involved in evasion of host cell death [50,60,61]. RID-α and RID-β are transmembrane proteins, while 14.7K is cytosolic. The central E3 proteins harbour conserved region 1 (CR1) domains and are termed CR1 proteins [62]. 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 [56]. 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 CPEs and cell lysis at the end of the viral replication cycle [29,63]. This was demonstrated by the formation of smaller plaques formed by an E3a-11.6K-deleted HAdV-C5/C2 (dl712) [29,64]. The deletion of other E3a or E3b genes did not affect the plaque size in cell cultures [64], with the exception of E1B-19K-deleted mutants, which caused larger plaques [65,66]. Accordingly,

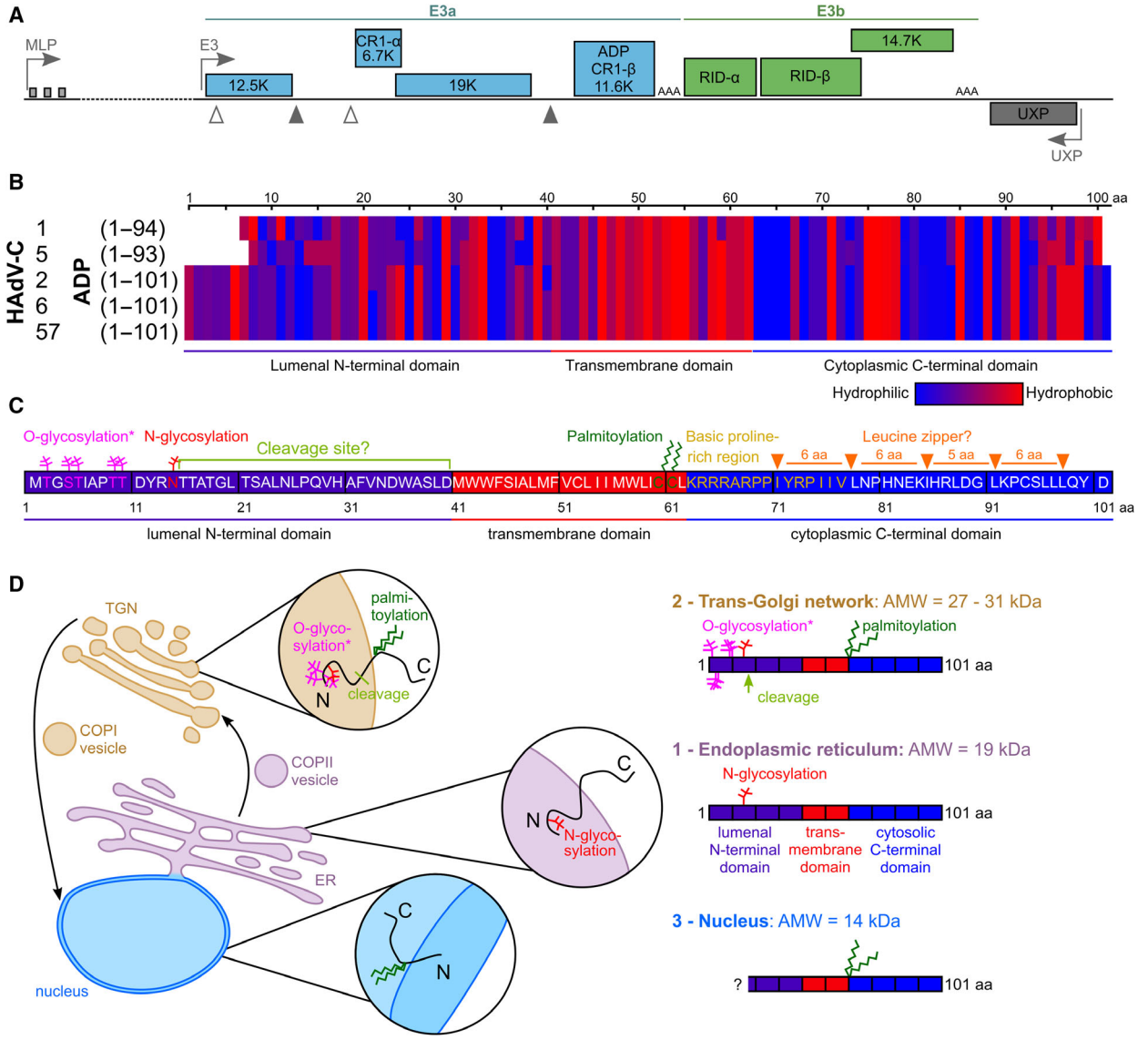

Fig. 2. The E3 transcription unit of HAdV-C and the processing of E3-11.6K/ADP. (A) The E3a transcription unit of HAdV-C2 (blue) encodes for the 11.6K protein ADP from the coding region CR1-β. E3b ORFs are shown in green. At early infection stages, ADP is expressed at low levels from the E3 promoter, represented as a grey arrow, located upstream of the E3 transcription unit. During late infection, ADP mRNA is transcribed from the 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 aa. The cytosolic domain further harbours hydrophobic aa (L and I) arranged in a leucine zipper-like pattern, indicated with orange triangles. (D) ADP, which is membrane-associated, is translocated from the ER via the TGN to the nucleus. Zoomed in graphics indicates 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 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.

HAdV-C2 E3A-11.6K was named adenovirus death protein (ADP) [29]. The corresponding 10.5K ADP of HAdV-C5 is slightly smaller than the C2 protein [63]. The C2 and C5 ADP exhibit 81% sequence identity. The functional 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 [67]. RL11 comprises 65–85 aa and a central conserved CXX(NQEKTY)$X_{4–6}$(YFLI)NX(ST)XXXXGXY motif (alternative residues in parentheses), 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 [56]. With a few exceptions, all CMV, CCMV and HAdV CR proteins contain a transmembrane domain and undergo N-terminal glycosylation. N- and O-glycosylation of CR1-γ (20.5K) in HAdV-B3 and B7, as well as an unusually large HAdV-D19 CR1-β of 49 kDa have been described [68,69]. 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 corresponding viruses [2,56]. 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 [56]. 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 [50]. The 6.7K protein localizes primarily to the endoplasmic reticulum (ER) membrane and represses apoptosis by interacting with RID-β [70]. 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 [71]. The CMV UL8 is a highly glycosylated late Ig-like protein that localizes to the cell surface [72]. It downmodulates 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 [73]. The deletion of its ortholog in HAdV-B3 did not affect progeny release from epithelial cells [73]. 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 (Fig. 2B).

The cell lytic function of ADP

Adenovirus death protein is expressed from the E3 promoter at low levels early in infection [74], when viral proteins affecting cell cycle regulation, inhibition of apoptosis, immune evasion and viral DNA replication are expressed [75,76] (see also Fig. 2A). Later in

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

HAdV species	HAdV type	NCBI reference sequence	Name	aa Sequence	aa Length	Weight (kDa)
C	1	AAQ10560.1	10.7 kDa protein	MVDTVNSYNTATGLTSTQDMPQVSTFVNNWANLG MWWFSIALMFVCLIIMWLSCCLKRKRARPPIY KPIIVLNPNNDGIHRLDGLNTCSFSFAV	94	10.7
C	2	AAA92222.1	11.6 kD protein	MTGSTIAPTTDYRNTTATGLTSALNLPQVHAFVND WASLDMWWFSIALMFVCLIIMWLICCLKRRRARPPIY RPIIVLNPHNEKIHRLDGLKPCSLLLQYD	101	11.7
C	5	AP_000221.2	10.5 kD protein	MTNTTNAAAATGLTSTTNTPQVSAFVNNWDNLG MWWFSIALMFVCLIIMWLICCLKRKRARP PIYSPIIVLHPNNDGIHRLDGLKHMFFSLTV	93	10.5
C	6	ACN88121.1	ADP glycoprotein CR1-beta0	MTGSTIAPTTDYRNTTATGLKSALNLPQVHAFVND WASLGMWWFSIALMFVCLIIMWLICCLKRRRARPPIY RPIIVLNPHNEKIHRLDGLKPCSLLLQYD	101	11.6
C	57	ADM46163.1	CR1 beta 11.6 kDa protein	MTGSTIAPTTDYRNTTATGLKSALNLPQVHAFVND WASLGMWWFSIALMFVCLIIMWLICCLKRRRARPPIYRP IIVLNPHNEKIHRLDGLKPCSLLLQYD	101	11.6

the viral replication cycle, when progeny virions are assembled, ADP is expressed at high levels from the major late promoter (MLP) [74]. This switch to high expression is facilitated by the L4-33K and L4-100K proteins [77]. MLP-driven ADP expression was found to be promoted by L4-22K [78], a protein involved in late viral gene expression, viral DNA packaging and progeny virion production [78]. 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 [28,29,64,79]. 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 RID-β deleted in VRX-006 and VRX-007 [28]. Alternatively, modification of a splice acceptor site upstream of ADP in HAdV-C5 expressing a mutator DNA polymerase leads to increased ADP expression from the E3 transcription unit [80]. 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 overexpressing HAdV-C5 viruses [81]. 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 [82,83]. This phenotype resembles the HAdV-C infection of epithelial or fibroblast cells in the presence of interferon, which suppresses the transcriptional activity of the E1A promoter [84,85]. Persistence-prone lymphoid cell lines indeed express lower levels of E1A and ADP [81], consistent with the notion that the E3 transcription unit is under the control of E1A [86]. However, overexpression of ADP in these lymphoid cell lines did not increase cell death, suggesting that additional factors are necessary to lyse these cells [81].

Nonlytic cell-to-cell transmission has been proposed for HAdV-F41, which causes gastroenteritis [2]. This was based on the observation that neutralizing antibodies only partly reduced HAdV-F41 transmission in cell cultures [87]. Remarkably, the genetic swap of HAdV-C5 ADP to the F41 E3 region resulted in a 10- to 50-fold increased release of cell-free HAdV-F41 progeny and a switch from slow-growing, symmetrical plaques to fast-growing comet-shaped plaques [88]. Symmetrical plaques are indicative of nonlytic cell-to-cell transmission [33]. The data confirm the unique lytic function of HAdV-C ADP and possibly accessory factors, and suggest that additional transmission mechanisms exist in HAdV infection.

Maturation of ADP

Adenovirus death protein is a type III integral membrane protein with a single transmembrane signal-anchor sequence (M_{41}-L_{62}) of 22 amino acids (aa) [89]. It localizes to the endoplasmic reticulum (ER), Golgi and inner nuclear membrane. With 101 aa, HAdV-C2 ADP is slightly longer than the 93 aa HAdV-C5 ADP (Fig. 2C,D). The transmembrane domain is essential for ADP function and cell lysis [90]. 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 [89]. In the ER, ADP is N-glycosylated cotranslationally at N_{14} [89], presumably by the oligosaccharyl transferase [91]. The glycosylation at N_{14} supports ADP transport to the trans-Golgi network (TGN) [89,90]. Transport likely occurs *via* the coatomer protein complex type II (COPII) machinery [92]. N-linked glycosylation generally supports protein folding and protects from proteasomal degradation [93–95]. 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 [90]. The O-linked glycans are thought to protect ADP from proteasomal degradation, as inferred from other O-glycosylated proteins in mammalian cells [96]. They are, however, only transiently associated with ADP due to proteolytic processing of ADP in the TGN between residues T_{10} and M_{41} [89]. Neither the exact cleavage site nor the processing protease has 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 [97].

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 aa preceded by R or K at position −4 [98]. S2P cleavage 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 [99,100]. 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 [90]. 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 [90]. The functions of the N-terminal proteolytic processing remain unknown, although N-terminal processing does not seem to affect cell lysis [90].

Furthermore, ADP undergoes cytosolic palmitoylation at C_{60} and C_{61} proximal to the transmembrane domain [101]. The processed palmitoylated ADP is transported back to the inner membrane of the nuclear envelope, likely *via* coat protein complex type I (COPI) vesicles [89]. This presents 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) [90]. 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} leads to the formation of TGN-like vesicles containing ADP [90]. 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 [102,103].

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 aa each [104]. 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 [105]. MAD2B is a TCF4-binding protein [106]. It localizes to the nucleus and modulates epithelial–mesenchymal transition [106]. MAD2B interacts with the DNA repair protein REV1 and the DNA polymerase ζ [107–111]. Yeast two-hybrid, GST pull-down and co-immunoprecipitation experiments showed that MAD2B interacts with HAdV-C2 ADP [104]. MAD2B binds to the cytosolic/nuclear C terminus 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 [104]. 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. The Nomenclature Committee on Cell Death 2018 acknowledges 12 distinct cell death pathways [112]. 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, for example by propidium iodide (PI) staining of the nucleus [113]. Necroptosis is a form of programmed necrosis, for example triggered by the activation of Toll-like receptors or virus infections [114,115].

HAdV-C5 infection induces cell death, which involves membrane permeabilization indicated by PI-positive nuclei [116]. This coincides with the release of progeny virions into the environment [28,30]. The inhibition of apoptosis by chemical inhibitors or overexpression of anti-apoptotic proteins did not reduce death of the infected cells [116,117]. 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 and thereby enables virus-controlled death [114]. 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 [30]. Yet, the virus evades innate immune responses and thereby maximizes the viral replication and progeny formation.

Human adenoviruses 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 and DNA degradation [116–125]. An early activator of apoptosis is the immediate early viral transactivator protein E1A, reviewed in White [126]. 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 & White [127]. 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 cytochrome C release.

Human adenoviruses-induced cell death also exhibits autophagic features [120,122,128–131]. Autophagic processes are induced by binding of E1B-19K to Beclin-1, also referred to as ATG6, one of the central regulators of autophagy [130,132]. 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 [118,133], and insights into the molecular mechanism were reported [134]. 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 may be involved in pan-adenoviral transmission.

HAdV-C infection further leads to a progressive loss of cell adhesion [135]. 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 [136]. In fact, viral capsomer proteins, including penton base and fibre, are released from the infected cell by a nonclassical pathway prior to lysis [137,138]. 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 antiviral response can lead to inflammatory tissue damage, and is exacerbated by caspase-mediated cleavage of pore-forming gasdermins [139].

ADP-mediated cell death

Ever since ADP was discovered [63], 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 [101,140,141]. 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 Fig. 2C. In this region, L, M, V and I are interspersed with 6–7 aa, typical of a quadruplicate leucine zipper motif [142,143]. Leucine zippers are known to induce dimerization or oligomerization of proteins [143]. Moreover, the proposed domain of ADP is preceded by basic amino acids, which may aid DNA binding [143]. 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 [29,144]. 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 [145]. 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 [89,90,101,146]. 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 [147,148]. Interestingly, ADP may counteract the apoptosis-suppressing function of E1B-19K [101] and thereby give rise to larger sized plaques and increased antitumour efficacy, as has been observed in E1B-19K-deleted HAdV [65,66,149]. Notably, E1B-19K is acylated (palmitoylated and myristoylated) and localizes to the ER and the nuclear envelope [150,151]. 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 [152,153]. Whether ADP synergizes with E1B-19K in inhibiting Bak/Bax remains to be investigated. Likewise, it is unknown whether ADP potentiates the pro-apoptotic effects of other early viral genes, such as E4orf4 [133,154–159].

ADP in cancer therapy

The selective removal 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 [160,161]. One of the first reported cases of cancer remission by a virus was from a woman receiving a live-attenuated rabies vaccine [162]. Subsequent clinical observations showed that sometimes virus infections correlated with cancer regression [163–165]. 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 [166,167]. This indicated that cytotoxic vectors are required to effectively cancer in immunocompetent patients. Coincidentially, the lytic nature of HAdV infection was discovered in 1953 [168,169] and this spurred intense interest to develop oncolytic therapy based on a variety of HAdV types [161,166].

Oncolytic HAdV-based vectors were used to investigate the role of HAdV proteins in tumour cell killing, reviewed in [170–173]. Regarding ADP [29], 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 [79]. To enhance tumour cell selectivity, they were equipped with two E1A mutations impairing E1A binding to Rb and p300 [174]. Tumour cell killing specificity was improved by replacing the E4 promoter by the promoter for surfactant protein B [175]. Both oncolytic viruses showed increased efficacy in xenograft models compared to wild-type HAdV-C5 [175]. Meanwhile, another ADP-overexpressing HAdV-C5-based oncolytic virus was generated in a comparable approach [176]. The E3 region with the exception of 12.5K of 01/PEME was replaced by a MLP-ADP overexpression cassette. 01/PEME was 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* [176]. 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 [125,177,178]. Also, the ADP-overexpressing Ad5-yCD/*mut*TK_{SR39}rep-ADP outperformed its parental E1B-55K- and ADP-deleted virus regarding antitumour effects and specificity *in vitro* and in xenograft models [179,180]. Taken together, ADP overexpression can potentiate the efficacy of oncolytic viruses.

However, severe host immune responses and limited tumour access are unresolved issues with oncolytic AdV in therapy [181,182]. It is clear that safety issues and enhanced tumour killing by AdV vectors will have to 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 [12,171,183–186].

Future questions in ADP research

Pathogen-associated molecular patterns of viruses 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 [187], the next frontier is to analyse the viral mechanisms of cell killing at the single-cell level. This will involve improved image-based approaches [76,188]. 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 [30]. Deep learning-enhanced image analyses allows predictions of infection outcome, for example lytic/nonlytic, and thereby reveals features of lytic or lysogenic cells [145]. Additionally, genetic manipulations have been significantly eased by the implementation of the CRISPR/Cas system [189]. In combination with high-throughput, genome-wide screening platforms [190], genotype–phenotype relations can today be addressed in an unbiased manner.

In conclusion, the ADP case can 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, and 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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Author contributions

FG prepared the figures and the table, and FG and UFG wrote the manuscript.

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