Microreview

Adenovirus signalling in entry

Nina Wolfrum and Urs F. Greber*
Institute of Molecular Life Sciences, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.

Summary
Viruses carry nucleic acids between and within host cells. Invariably, virus attachment to host cells leads to activation of cell signalling. These so-called forward signals emerge from interactions with cell surface receptors or cytosolic proteins and elicit profound responses in the cells, for example induction of growth or innate immunity responses. They can enhance or suppress infection. In addition, viruses receive signals from the cell. These reverse signals can impact on the structure of the virus leading to genome uncoating. They can enhance infection or inactivate virus, for example by facilitating degradation. Here we discuss the nature and mechanisms by which forward and reverse signals emerge and affect the outcome of human adenovirus infections. We describe how human adenoviruses use cell surface receptors for forward signalling to activate cell growth, intracellular transport or innate immune response. We also discuss how adenoviruses use acto-myosin, integrins or microtubule-based kinesin motors for reverse signalling to facilitate their stepwise uncoating programme.

Introduction
Viruses are diverse in nature and appearance. They range from small non-enveloped single-stranded RNA or DNA viruses to lipid bearing double-stranded RNA or DNA viruses with haploid or diploid genomes. Despite large diversity, they share common principles enabling infection. One invariable feature is that they protect their genome with a proteinaceous capsid and sometimes a lipid envelope. Capsid or envelope proteins dock to cellular receptors or soluble proteins. Another conserved feature between viruses is that they activate cell-signalling processes as soon as they interact with cells. In some cases this leads to the stimulation of endocytic uptake processes, such as clathrin-mediated endocytosis or macropinocytosis (Amstutz et al., 2008; Gastaldelli et al., 2008; Mercer and Helenius, 2009), the interference with the cell cycle or induction of cancer (Damania, 2007). Many aspects of adenovirus (AdV) biology have been investigated, and rapid progress occurs in areas of virus structure and entry, replication, pathology and immunology, evolution, vector development and vaccinations (recently reviewed in Greber et al., 2012). Here we discuss how human adenoviruses (HAdV) induce signalling to the cell during entry, and how the viruses receive signals from the cell by a reverse process.

Forward signalling from cell surface receptors
Cell surface receptors mediate initial contacts of viruses to cells, and this can trigger cell signalling. If virus–receptor interactions are of high affinity, a few hundred receptor molecules per cell are sufficient for infection. If the interactions are of low affinity (in the micromolar or even millimolar range), or if the binding site on the receptor is masked, increased numbers of receptors are required for virus attachment. This can occur through avidity binding. Viruses are able to use avidity binding since they often display multiple receptor binding sites in close spatial proximity. In the case of HAdVs, this has been demonstrated for binding of HAdV-3/7 to the membrane cofactor CD46 (Trinh et al., 2012). Furthermore, C-species HAdV (HAdV-C) also bind with a large spectrum of affinities to their receptor, the coxsackievirus adenovirus receptor (CAR) (Kirby et al., 2001). Low-affinity, high-avidity binding allows viruses to use multiple receptor binding sites in close spatial proximity. In the case of HAdVs, this has been demonstrated for binding of HAdV-3/7 to the membrane cofactor CD46 (Trinh et al., 2012). Furthermore, C-species HAdV (HAdV-C) also bind with a large spectrum of affinities to their receptor, the coxsackievirus adenovirus receptor (CAR) (Kirby et al., 2001). Low-affinity, high-avidity binding allows viruses to use multiple receptors depending on receptor availability, and this may broaden virus tropism. In the following section, we discuss the current picture of attachment factors, entry receptors, and supporting bridging factors for HAdV infections (for an overview, see Fig. 1).

The coxsackievirus adenovirus receptor CAR
AdV were first isolated in the 1950s (Rowe et al., 1953). However, the identification of the first AdV receptor

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CAR took more than 40 years (Bergelson et al., 1997; Tomko et al., 1997). CAR is involved in the formation of tight and adherens junctions between epithelial cells (Cohen et al., 2001; Walters et al., 2002). It belongs to the immunoglobulin superfamily and is expressed in heart, brain, pancreas, intestine and at low levels in liver and lung (Fechner et al., 1999). CAR interacts with the fibre knobs from HAdV of all species, except those from species HAdV-B (Roelvink et al., 1998). Interestingly, the HAdV fibres bind to the same extracellular domain of CAR (the D1 domain), which is also engaged in homophilic CAR–CAR contacts between neighbouring cells, however, with much higher affinity (Bewley et al., 1999; Freimuth et al., 1999). In contrast to CAR dimerization, the attachment of viruses to CAR has been reported to elicit intracellular signalling that leads to an inflammatory response and expression of cytokines in human respiratory cells (Tamanini et al., 2006). In addition, CAR overexpression studies in epithelial cells suggested that CAR is directly or indirectly involved in the activation of p42/44 extracellular receptor kinases (ERK) and could enhance β1/3 integrin expression (Farmer et al., 2009). The cytoplasmic domain of CAR appears to be involved in ERK activation (Farmer et al., 2009), but can be dispensable for HAdV cell entry (Wang and Bergelson, 1999; Burckhardt et al., 2011). This is consistent with chemical interference studies showing that ERK signalling is not required for virus entry but rather plays a critical role in the inflammatory process triggered by the virus (Bruder and Kovesdi, 1997; Suomalainen et al., 2001; Smith et al., 2011).

The membrane cofactor CD46

How HAdV-B species bind to blood and epithelial cells has remained unknown until recently. In 2003/04, four groups reported that the membrane cofactor CD46 was required for binding of HAdV-3, 11, 16, 21, 35, 50 (species HAdV-B1 and B2), as well as HAdV-37 (species HAdV-D) to epithelial and haematopoietic cells (Gaggar et al., 2003; Segerman et al., 2003; Sirena et al., 2004; Wu et al., 2004). More recently, HAdV-26 and 49 (species HAdV-D) were also found to use CD46 as a receptor (Lemckert et al., 2006; Li et al., 2012). These studies showed that all HAdV-B attach to cells via their fibre knobs, which recognize the extracellular domain of CD46, albeit with different affinities (Pache et al., 2008; Cupelli et al., 2010). This was concluded from a variety of biochemical and cell biological experiments and infection assays, including cDNA expression in receptor-negative non-human cells, virus–receptor colocalizations at the ultrastructural level, in-solution binding assays, mass spectrometry, antibody inhibitions of cell binding and infection, competition experiments with soluble fibre or receptor domains, or RNA interference in cultured or primary cells. CD46 is expressed on all nucleated cells in humans, and shields autologous cells from complement attack (for a review, see Riley-Vargas et al., 2004).

| Tropism  | Entry receptor | Bridging factors |
|----------|----------------|------------------|
| Respiratory | CAR | CD46 | DSG2 | SA | GD1a | Integrins | HSPG | FX | DPPC | Lf |
|          | 2, 4, 5, 12, 15, 19p, 31 | 3, 7, 14, 16, 21, 35 | 3, 7, 14 | 2, 3, 5, 35 | 2, 3, 5 | 2, 5 | 2, 5 |
| Ocular   | 5, 9 | 37, 49 | 8, 19a, 37 | 8, 19a, 37 |
| Renal    | 11, 35 | 11, 14, 35 |
| Intestinal | 41 |
| Liver    | 2, 3, 4, 5, 6, 7, 8 | 2, 5, 17, 24, 30, 33, 45, 47 |

Coxsackie and adenovirus receptor (CAR), Desmoglein 2 (DSG2), Sialic acid (SA), Ganglioside 1a (GD1a), Heparan sulfate proteoglycan (HSPG), Coagulation factor X (FX); Dipalmityl phosphatidylcholine (DPPC); Lactoferrin (Lf)

Colour-code for the adenovirus (sub)-species: A, B1, B2, C, D, E, F

Fig. 1. Summary of HAdV entry receptors, attachment and bridging factors and tropism. Additional information and literature can be found in the main text.
Interestingly, ligand-induced CD46 oligomerization triggers macropinocytosis and downregulation of CD46 (Crimeen-Irwin et al., 2003), which in turn increases immune suppression and complement-mediated lysis. HAdV-3/35 (HAdV-B species) use CD46 for infection, and they enter and infect epithelial and haematopoietic cells by triggering macropinocytosis (for an overview of the pathway, see Fig. 2, and Amstutz et al., 2008, Kalin et al., 2010). These viruses also use the C-terminal binding protein 1 of E1A (CtBP1), which is a phosphorylation target of p21-activated kinase (PAK) 1. CtBP1 is a multifunctional protein involved in transcriptional repression and membrane trafficking, including PKA1-mediated macropinocytosis (Liberali et al., 2008). HAdV-3 entry activates PKA1 through Rac1, and both PKA1 and Rac1 are required for HAdV-3 or 35 infections (Amstutz et al., 2008; Kalin et al., 2010). Macropinosomes positive for fluorescent fluid phase marker dextran contain viruses and CD46, \( \alpha_v \beta_3/5 \) integrin, CtBP1 and PAK1. Collectively, the data show that HAdV-B bind to CD46 and induce an endocytic pathway, which is used in antigen presentation and cell migration in non-polarized cells. The HAdV-B entry pathway may modulate immune responses possibly by interference with the transcriptional co-repressor CtBP1. Whether CD46 has a receptor function for HAdV-B on polarized cells lining tissue epithelia has not been reported so far.

**The cell adhesion molecule desmoglein 2**

Based on cross-competition data between HAdV-B types in different cells and neutralizations of infections with fibre knobs, it was widely assumed that there are additional attachment receptors for HAdV-B species, besides CD46. Using blot overlay techniques on SDS-denatured gel fractionated extracts from receptor positive and negative cells in combination with mass spectrometry, desmoglein-2 (DSG-2) was identified as an attachment receptor for HAdV-3, 7, 11, 14 (Wang et al., 2011). DSG-2 belongs to the cadherin family of calcium-binding trans-membrane glycoproteins and is a component of the adhesion complexes between epithelial cells. The affinities of fibres to DSG-2 are weak, as the trimeric fibre knob from HAdV-3 did not bind to DSG-2, unlike intact HAdV-3 or penton-fibre complexes, so-called dodecahedrons. This was similar to CD46, which bound to HAdV-3 or dodecahedrons by an avidity mechanism (Sirena et al., 2004; Trinh et al., 2012).

Intriguingly, the binding of AdV or dodecahedrons to DSG-2 elicited events similar to epithelial-to-mesenchymal transition (EMT), as evidenced by marker profiling and p42/44-ERK or phosphatidylinositol-3 kinase phosphorylations (Wang et al., 2011). Virus-induced EMT apparently opened up intercellular junctions. In the context of an epithelium, this could lead to the exposure of basolateral receptors towards the luminal side, for example the airway lumen, and thereby enhance the accessibility of the epithelium to exogenous agents and enhance infection. This is reminiscent of earlier reports showing that the release of excess fibre proteins from AdV-infected cells disrupted cell–cell contacts and promoted the release of newly synthesized viruses from epithelia (Walters et al., 2002).
Sialic acid and bridging factors

Highly abundant structures in glycoproteins or glycolipids of the cell surface are the negatively charged sialic acids. HAdV-8, 19, 37 (species HAdV-D) contain positive charges in their fibre knobs and bind sialic acid (Nilsson et al., 2011). These interactions, however, do not account for the ocular tropism of species HAdV-D, since sialic acid is not specific for cells of the conjunctiva. Possibly, virus interactions with sialic acid contribute to AdV haemagglutination, given that the erythrocyte surface is rich in the sialic acid bearing glycoporphin A. It is also possible that virus attachment to sialic acid enhances the binding to other low-affinity attachment sites, from which signals are transduced to the host cell.

Other low-affinity attachment sites for AdV are heparan sulfate-containing proteoglycans (HSPG) of the extracellular matrix (ECM). They comprise trans-membrane syndecans and glycosyl-phosphoinositol-linked glypicans. HSPG were reported to bind the fibre shaft of HAdV-C through a lysine-lysine-threonine-lysine (KKTK) motive (Dechecchi et al., 2001; Darr et al., 2009). Recently, HSPG was suggested as an attachment site for mouse AdV-1 on endothelial cells (Lenaerts et al., 2012), and for assisting HAdV-3 attachment to integrin (Tuve et al., 2008; Gout et al., 2010). The precise role of HSPG in AdV infection remains elusive, however, also because the affinity of AdV to HSPG is apparently much lower than of Herpes simplex virus type 1, which uses HSPG for infection (Shukla et al., 1999; Kalin et al., 2010). Accordingly, little information exists for potential signalling events from bridging factors in HAdV entry. For further information on coagulation factor X, lung surfactant di-palmitoyl-phosphatidylcholine or lactoferrin, the reader is referred to Fig. 1, and a review (Arnegb, 2009).

Integrins as signalling receptors

Integrins are adhesion receptors, consisting of an α and β subunit, and connect cells to the ECM. They transduce outside-in or inside-out signals, and regulate cell survival and migration. Integrins were discovered as AdV receptors long before CAR was known. An initial key observation was that soluble penton base from HAdV-2 precluded the attachment of αv integrin-positive cells to culture dishes (Wickham et al., 1993). Soluble penton base did not interfere with virus attachment to cells but inhibited virus endocytosis, similar to soluble RGD (arginine-glycine-aspartate) peptides, a motif also present in penton base. The RGD motive is found in all HAdV penton bases, except HAdV-40/41 (species HAdV-F), allowing these viruses to use αvβ3, αvβ5, α5β1, or αvβ2 integrins depending on the cell type (reviewed in Stewart and Nemerow, 2007).

The best-characterized integrins in HAdV entry are αvβ3 and αvβ5 expressed on many epithelial cells (for a schematic drawing, see Fig. 3A). Both of them promote virus internalization, depending on the penton base RGD or an engineered RGD in the fibre knob (Wickham et al., 1993; Nagel et al., 2003). This suggests that HAdV use integrins that are in an activated state, and transduce signals, for example to phosphatidylinositol-3-OH kinase (PI3K) (Li et al., 1998b). Lipid products of PI3K can activate the small GTP-binding proteins RhoA, Rac1 and Cdc42 and lead to actin remodelling, which is important for viral endocytosis (Li et al., 1998a). These data suggest a positive feedback loop between integrins, PI3K and actin to promote virus uptake (Fig. 3A).

The uptake of HAdV-2/5 is controlled by the large GTPase dynamin (Wang et al., 1998; Meier et al., 2002; Gastaldelli et al., 2008). Recent proteomics analyses have shown that HAdV-5 entry leads to S-nitrosylation of dynamin-2 at cysteine residues C86 and C607, and that the overexpression of S-nitrosylation-resistant C86A or C607A dynamin mutants reduces HAdV infection (Wang et al., 2012). S-nitrosylation is likely mediated by eNOS (endothelial nitric oxide synthase), which gets activated by denitrosylation and phosphorylation upon HAdV-5 entry (Fig. 3A). It remains unknown, however, how HAdV activates eNOS and increases nitric oxide (NO) in epithelial cells.

Signalling to increase receptor availability

The respiratory, digestive, urogenital and ocular tracts are major portals of entry for viruses into vertebrates. Polarized epithelial cells provide the borders for tissues, typically with their apical plasma membrane facing the outside. Intriguingly, many receptors for viruses are not localized on the apical membrane, but at the basal and lateral membranes. This renders polarized cells resistant against infections, for example by HSV-1, poliovirus, reovirus or HAdV (Greber and Gastaldelli, 2007; Bergelson, 2009). The reason why these viruses have no apical receptors is unclear. One can speculate that it is due to protection of the apical membrane by a mucus layer, which precludes the accessibility of the apical plasma membrane to agents in the lumen of the respiratory tracts, for example.

HAdVs invade the lumen of the digestive and respiratory tracts. Their receptors CAR, DSG-2 and integrins are localized at the basolateral membranes. In the airway lumen, alveolar macrophages engulf incoming viruses. This triggers the release of chemotactic and pro- and anti-inflammatory cytokines (Zsengeller et al., 2000; Higginbotham et al., 2002). The released cytokines include junction-remodelling factors, such as interferon (IFN) γ and tissue necrosis factor alpha (TNF-α). Junctional remodelling by cytokines may facilitate the passage of activated immune cells across the epithelial border (Zen et al., 2005).
A. Forward signalling and receptor accessibility. HAdV-2/5 can be taken up by macrophages that “patrol” on the apical side of polarized epithelial cells. This leads to the secretion of various cytokines including interleukin 8 (IL-8). IL-8 binds to the CXCR1/2 receptor and triggers the phosphorylation (P) of the non-receptor tyrosine kinase Src and the adaptor paxillin (PXN). These proteins are involved in relocating CAR and integrins from the basal to the apical surface and thus enable virus attachment and infection from the apical plasma membrane (Lutschg et al., 2011). The binding of HAdV-2/5 to integrins leads to numerous intracellular signals, including PI3K and the actin remodelling GTPases RhoA, Cdc42 and Rac, and promote virus endocytosis (see main text). HAdV-2/5 endocytosis is controlled by dynamin-2 (Dyn2), which gets S-nitrosylated (NO) most likely by the endothelial nitric oxide synthase (eNOS) (Wang et al., 2012). This pathway leads to infection.

B. Reverse signalling. Reverse signals from the cell to the virus emerge for example, when myosin2 and actin-dependant movements of CAR are attenuated by immobile integrins. This creates a force on the virus, and leads to loosening of the capsid structure, as measured by the loss of fibres (upper part) (Burckhardt et al., 2011). Additional reverse signals from the cell to the virus may come from cytoplasmic motors, such as dynein/dynactin and result in displacement of the cytosolic virus towards the nucleus. We do not show these events, since they have been covered in recent reviews (Greber and Way, 2006; Dodding and Way, 2011; Scherer and Vallee, 2011). During later steps of infection the virus docks to the nuclear pore complex (NPC) by interacting with the nucleoporin (Nup) 214, and subsequently binds to the light chain (Klc1/2) of the conventional kinesin Kif5C (Strunze et al., 2011). The movement of the motor towards the microtubule plus end occurs against a holding force from the NPC, and leads to the disruption of the capsid and in part the NPC. This facilitates the release of the viral DNA into the nucleus (lower part).
A recent study has shown that human blood-derived macrophages inoculated with HAdV-2 release IFN-γ, TNF-α or interleukin 6 (IL-6), and also the chemokine IL-8 (Lutschg et al., 2011). When polarized epithelial airway cells were co-cultured with macrophages, they become susceptible to apical HAdV-2. Susceptibility depends to a large extent on IL-8 released from the macrophages. The treatment of polarized epithelial cells with IL-8 was sufficient to induce the expression of CAR and αvβ3 integrins on the apical surface, while IFN-γ had no effects (see Fig. 3A). This suggests that CAR and αvβ3 integrins are associated with cell migration and immune signalling. IL-8 strongly enhanced apical infection of polarized cells by signalling through the G-protein coupled chemokine receptors CXCR1/2, thereby activating the non-receptor tyrosine kinase Src and the adaptor paxillin. The observation that CAR, which normally engages in homophilic contacts between neighbouring cells, delocalized to the apical membrane under inflammatory conditions suggests that it directly or indirectly co-ordinates inflammatory responses (Verdino et al., 2010). One can speculate that those HAdV, which elicit low levels of inflammatory responses, are less dependent on cytokines for epithelial infections. These viruses might have found ways to infect epithelial cells by using apical attachment factors, such as CD46, particular lipids, sugars or bridging factors (see also Fig. 1).

**Reverse signalling**

Virus entry involves a two-way dialogue between the virus and the cell, and comprises forward signalling from the virus to the cell and reverse signalling from the cell to the virus. Already, directly upon AdV attachment to the cell, the particle starts moving on the cell surface. These CAR-dependent motions comprise rapid diffusive motions and steady acto-myosin-dependent drifts (Burckhardt et al., 2011). The drifts are based on actin filaments moving retrograde from the periphery to the cell body. They are driven by actin polymerisation at the periphery and pulling forces from myosin-2 (for a review, see Burckhardt and Greber, 2009).

The drifting motions of CAR are of key importance for triggering initial steps of the virus-uncoating programme, which starts with the release of the fibres from the virus (Fig. 3B, upper panel). This is assisted by another reverse signal, the binding of αvβ3 integrins to the virus penton base (Burckhardt et al., 2011). Unlike CAR, integrins are static receptors and their motions at the surface are confined. If CAR-induced movements and integrin-induced confinements occur simultaneously at one virus particle, the resulting force on the virus contributes to loosening the virus structure. This loosening could be enhanced by the interaction of the RGD-loops from penton base with integrins. The latter lead to a clockwise rotation of the penton base pentamer (Lindert et al., 2009). These opposed forces applied to the virus at the cell surface also enable the exposure of protein VI from the inside of the virus. During later steps of infection this can enhance the hydrophobic surface of the virus to break open the limiting endosomal membrane (Wiethoff et al., 2005; Burckhardt et al., 2011).

Upon endosomal escape, the AdV particle is transported via dynein along microtubules towards the perinuclear region. This process was recently reviewed (Dodding and Way, 2011; Scherer and Vallee, 2011). Another event of reverse signalling occurs at one of the last steps of the HAdV-2/5 uncoating programme, the disruption of the capsid and the dissociation of the viral DNA from the capsid (see Fig. 3B, lower panel). This takes place at the nuclear pore complex (NPC) (Trotman et al., 2001), and requires the microtubule motor Kif5C (Strunze et al., 2011). Upon docking at the nucleoporin (Nup) 214, the virus recruits conventional kinesins by binding to the Kif5C light chain Klc1/2 (Strunze et al., 2011). The heavy chain is pre-associated to Nup358 at the NPC. Kif5C binding to Nup358 stabilizes the open conformation and leads to allosteric motor activation (Cho et al., 2009). For virus disruption, Kif5C is thought to exert a force on the capsid against a holding force from the NPC. This leads to the separation of the viral DNA from the capsid, and kinesin motors and virus capsid fragments move from the NPC to the cell periphery (Strunze et al., 2011). Now the DNA in association with protein VII but not protein V can be imported into the nucleus, which involves nuclear import factors, such as transportin, importin beta and importin 7 (Trotman et al., 2001; Hindley et al., 2007). This is an example for a direct back-signalling process from the cell to the virus with consequences for virus and NPC integrity. It ensures that the uncoated DNA can readily access the nuclear translocation machinery. We anticipate that other back-signalling events take place on endosomal or cytosolic AdV particles, and possibly also non-related viruses.

**Conclusions and outlook to signalling for innate responses**

It has been well established that incoming HAdV trigger signal transduction pathways leading to cell activation and innate immunity. The latter processes are less well characterized than the cell activation signals (Fejer et al., 2011). For example, binding of the HAdV-5 fibre to CAR on epithelial cells leads to NFκB activation and enhances the transcription of the chemokines IL-8, GRO-α, GRO-γ or RANTES (Tamanini et al., 2006). p42/44-ERK or p38 mitogen-activated protein kinase appear not to be
involved, but HAdV DNA and particular sequence motifs therein are decoded in endosomes by Toll-like receptor 9 (TLR9) leading to innate signalling (Iacobelli-Martinez and Nemerow, 2007; Perreau et al., 2012).

The rupture of endosomal membranes and residence of viruses in the cytosol also contribute to innate immunity signalling. This was suggested from experiments with the HAdV-2 mutant ts1, which is unable to break endosomal membranes and therefore cannot reach the cytosol (Tibbles et al., 2002; Imelli et al., 2009; Smith et al., 2011; Maier et al., 2012). Various cytosolic sensors have been implicated in innate signalling during AdV entry (Fejer et al., 2008). The nucleotide oligomerization domain (NOD)-like receptor (NLR) containing pyrin domain 3 (NLRP3) triggers an inflammasome-dependent response, leading to activation of the IL-1 receptor, NFκB and chemokine release (Barlan et al., 2011). Furthermore, NOD2, another NLR exclusively expressed in monocytes, macrophages, dendritic cells and intestinal Paneth cells, was shown to be involved in innate immune response to HAdV vectors (McDermott and Tschopp, 2007; Suzuki et al., 2011). In a previous study it was already demonstrated that NOD2 responds to single-stranded RNA (Sabbah et al., 2009). Absent In Melanoma (AIM) 2 and the helicase DDX41 are involved in activation of the IFN response factor 3 (Stein and Falck-Pedersen, 2012). To antagonize an anti-viral state, the immediate early trans-activator E1A of HAdV-5 binds to and dissociates the hBre1/RNF20 ubiquitin ligase complex, thus inhibiting mono-ubiquitination and inducing the suppression of interferon-stimulated genes ( Fonseca et al., 2012). It will now be interesting to investigate, how modulations of host anti-virus responses are integrated in the overall host transcriptional response to HAdV infections. This may lead to a better understanding of both anti- and pro-viral host response pathways.

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