The E3 Ubiquitin Ligase Mind Bomb 1 Controls Adenovirus Genome Release at the Nuclear Pore Complex

Graphical Abstract

Highlights
- Genome-wide RNAi screen identifies host factors boosting or inhibiting AdV entry
- The E3 ubiquitin ligase Mib1 enhances entry of AdVs from different species
- Ubiquitination activity of Mib1 licenses AdV uncoating at the nuclear pore complex
- Mib1 at NPC-docked AdVs releases viral DNA into nucleus and cytosol “on demand”

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In Brief
Adenoviruses infect multiple organs in humans, delivering their DNA genome for replication to the nucleus. Adenovirus vectors are widely used. Bauer et al. identify a mechanism for virion DNA uncoating at the nuclear pore complex, licensed by the E3 ubiquitin ligase activity of Mind bomb 1.

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The E3 Ubiquitin Ligase Mind Bomb 1 Controls Adenovirus Genome Release at the Nuclear Pore Complex

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SUMMARY

Adenoviruses (AdVs) cause respiratory, ocular, and gastrointestinal tract infection and inflammation in immunocompetent people and life-threatening disease upon immunosuppression. AdV vectors are widely used in gene therapy and vaccination. Incoming particles attach to nuclear pore complexes (NPCs) of post-mitotic cells, then rupture and deliver viral DNA (vDNA) to the nucleus or misdeliver to the cytosol. Our genome-wide RNAi screen in AdV-infected cells identified the RING-type E3 ubiquitin ligase Mind bomb 1 (Mib1) as a proviral host factor for AdV infection. Mib1 is implicated in Notch-Delta signaling, ciliary biogenesis, and RNA innate immunity. Mib1 depletion arrested incoming AdVs at NPCs. Induced expression of full-length but not ligase-defective Mib1 in knockout cells triggered vDNA uncoating from NPC-tethered virions, nuclear import, misdelivery of vDNA, and vDNA expression. Mib1 is an essential host factor for AdV uncoating in human cells, and it provides a new concept for licensing virion DNA delivery through the NPC.

INTRODUCTION

Molecular interactions in virus-infected cells control the outcome of infection. Signaling events from the virus particle (virion) to the cell affect, for example, nutrient-sensing pathways, cell cycle progression, growth, migration, anti-viral immunity, cell survival, and death. In turn, virions receive chemical and mechanical cues from the invaded cell and engage a controlled, stepwise destabilization process that culminates in uncoating of the genome (Wolfrum and Greber, 2013; Yamauchi and Greber, 2013). In most cases, dismantling starts upon receptor binding at the plasma membrane and continues in endosomes, the cytoplasm, and the nucleus until the genome is completely uncoated (Flatt and Greber, 2017; Marsh and Helenius, 2006; Wang et al., 2018; Witte et al., 2018).

Viruses deliver their genome into the nucleus of post-mitotic cells by hijacking the nuclear import machinery and the nuclear pore complex (NPC). The NPC provides a size-limited passageway between the cytoplasm and the nucleoplasm (Kim et al., 2018; Schmidt and Görlich, 2016). It restricts import of most viral capsids, with the exception of very small capsids, such as parvoviruses and circoviruses (Kobiler et al., 2012). Hepatitis B virus with a similarly small capsid is thought to enter the NPC and dissociate its closed circular and partly double-stranded DNA within the NPC using a spring-loaded mechanism (Dhason et al., 2012). Virion mechanics are implicated in releasing the pressurized DNA genome from herpesvirus capsids and also entropically confined genomes in adenoavirosis (AdV) particles at the NPC (Greber, 2016; Shahin et al., 2006).

AdVs are widespread and cause a variety of infections in vertebrates, including fish, reptiles, rodents, birds, non-human primates, and humans (Davison et al., 2003). Human AdVs infect the respiratory and gastrointestinal tracts, kidneys, liver, eyes, as well as blood cells and persist in lymphoid cells of the digestive tract and can cause inflammatory disease (Cook and Radke, 2017). Human AdV species C type 2 or 5 (AdV-C2/5) particles enclose a linear double-stranded DNA of 36 kbp in an icosahedral capsid of ∼90 nm. In the stepwise entry process, they shed fibers and pentons, expose membrane lytic protein VI, rupture the endosome, and engage in cytoplasmic transport to the nuclear envelope, docking to the NPC and releasing the genome for nuclear import along with protein VII, which is assembled with the viral DNA (vDNA) in a nucleosome-like structure (Burckhardt et al., 2011; Cassany et al., 2015; Greber et al., 1993; Luisoni et al., 2015; Moyer et al., 2011; Os- tapchuk et al., 2017; Trotman et al., 2001; Wang et al., 2017; Wiethoff et al., 2005). The partly dismantled virions rupture at the NPC by mechanical force from microtubule-dependent motors (Strunze et al., 2011), release the genome to the nucleus for transcription, but also misdeliver vDNA to the cytosol (Puntener et al., 2011; Wang et al., 2013). Misdelivered DNA is decoded by cytosolic DNA sensors, such as cGAS and the inflammasome (Lam et al., 2014), which explains, at least in part, the inflammatory nature of AdV infection in a broad range of cell types, including immune cells (Greber and Flatt, 2019). The intricate
Figure 1. Mib1 Is an Essential Host Factor for AdV Infection

(A) Overview of the experimental workflow of the genome-wide siRNA screen in HeLa cells. The infection Z scores from the genome-wide screen are plotted per gene in ascending order. The Z score of Mib1 is indicated. See also Tables S1, S2, and S3. (B) A549 cells transfected with siRNAs against GFP, Mib1 or a non-targeting control were infected with AdV-C5-GFP at MOI 0.3 for 24 h. Nuclei were stained with DAPI, cells were analyzed by fluorescence microscopy, and infection was scored by measuring the GFP signal over the nuclei. The mean ± SD is shown (n = 3 replicates). Statistical significance was assessed using one-way ANOVA with Holm-Sidak correction for multiple comparisons.

(legend continued on next page)
entry process involves a plethora of host factors, most of which are unknown. Here, we conducted a genome-wide RNAi screen in human cells infected with AdV-C2. We identified a novel licensing factor for viral genome uncoating at the NPC, the E3 ubiquitin ligase Mind bomb 1 (Mib1), and show that the ligase activity controls nuclear import and cytoplasmic misdelyivery of vDNA from AdV-C2/5.

RESULTS AND DISCUSSION

Genome-wide RNAi Screen against AdV Infection

In search of new host factors involved in AdV entry and gene expression, we performed an arrayed genome-wide, image-based small interfering RNA (siRNA) screen using four siRNAs combined per gene in HeLa cells. Dissecting the molecular functions of cells by RNAi screens is a powerful but inherently noisy procedure with off-target effects because of cell toxicity of the siRNAs or their functioning as microRNAs with a broad range of targets (Daga et al., 2018; Franceschini et al., 2014).

Genome-wide RNAi screens have been reported for a range of viruses, including HIV, hepatitis C virus, Ebola virus, West Nile virus, vaccinia virus, influenza A virus, papillomavirus, enterovirus 71, bunyavirus, Semliki Forest virus, rotavirus, and adenov-associated virus but not AdV (Balistreri et al., 2014; Goff, 2008; Green and Pelkmans, 2016; Hao et al., 2013; Karlas et al., 2010; Krishnan et al., 2008; Li et al., 2009; Lipovsky et al., 2013; Mano et al., 2015; Meier et al., 2014; Smith et al., 2010; Su et al., 2013; Watanabe et al., 2010; Wu et al., 2016).

Here we provide the first genome-wide RNAi screen for AdV. Cells were infected with AdV-C2 encoding GFP under a cytomegalovirus (CMV) promoter in place of the E3B gene region (AdV-C2-ΔE3B-CMV-GFP) at an MOI of 0.1 (Yakimovich et al., 2012), stained with DAPI and phalloidin to label cytoplasmic F-actin, imaged in a high-throughput microscope, and analyzed for GFP expression 16 h post-infection (pi) (Figure 1A). The procedure was similar to a previous siRNA screen of the human kinome with a range of pathogens, including AdV-C2-ΔE3B-CMV-GFP (Ramó et al., 2014). 

$Z$ score analyses of 16,462 host genes indicated a continuous distribution of hits; 159 siRNA pools gave $Z$ scores smaller than $-2$ (inhibiting infection) and 440 larger than $+2$ (enhancing infection) in an overall range from $-2.99$ to $+6.51$ (Figure 1B; Table S1). The top 500 siRNAs downregulating infection gave $Z$ scores lower than $-1.57$ (Table S2). Grouping these hits into functional clusters using STRING (Szklarczyk et al., 2017) gave low false discovery rates smaller than $10^{-4}$ in the case of the COP-I-coated vesicle machinery, NPC components, the mediator complex, the spliceosomal complex, cytosolic ribosomes, preribosomes, or the nucleolus, which are directly or indirectly involved in gene expression and protein production (Figure S1A; Berk, 2005; Vijayalingam and Chinnadurai, 2013). The top 500 siRNAs upregulating infection had $Z$ scores higher than $+1.91$ (Table S3) and comprised broadly defined clusters, such as endo-lysosomal membranes, intracellular organelles, or protein-protein complexes with high false discovery rates between 0.01 and 0.05. These hits may reflect the fact that HeLa cells have a compromised anti-viral defense; for example, because of expression of the human papillomavirus E6 and E7 proteins, which antagonize p53, pRb, or cyclic guanosine monophosphate (GMP)-AMP synthase (Figure S1B; Lau et al., 2015; Mantovani and Banks, 2001; Münger et al., 2001).

The E3 Ubiquitin (Ub) Ligase Mib1, Controlling Developmental Signaling and Innate Immunity, Is Broadly Required for AdV Infection

We analyzed siRNA hits downregulating AdV infection, particularly those not affecting host gene expression and protein synthesis, and focused on one of the strongest hits in our validation, the E3 Ub ligase Mib1. Mib1 is a RING-type E3 Ub ligase of 1,006 amino acids, first discovered in zebrafish and highly conserved in almost all higher organisms (Deshales and Joazeiro, 2009). It localizes to centriolar satellite complexes on microtubules in the vicinity of the centrosome (Villumsen et al., 2013). Ubiquitination can have a strong effect on the structure, function, localization, and stability of the targeted protein, and viruses have evolved to exploit ubiquitination (Komander and Rape, 2012; Luo, 2016). For example, the Ub-proteasome system has been implicated in entry of dengue virus, Kaposi’s sarcoma-associated herpesvirus, vaccinia virus, and influenza virus (Banerjee et al., 2014; Dejarnac et al., 2018; Greene et al., 2012; Gschweitl et al., 2016; Mercer et al., 2012; Su et al., 2013). Human AdVs have also been shown to manipulate the cellular ubiquitination machinery during replication (Orazi et al., 2011; Querido et al., 2001; Schreiner et al., 2010). However, a role of a specific E3 Ub ligase in virion dismantling has remained elusive.

Our RNAi against the E3 Ub ligase Mib1 strongly reduced AdV infection but not cell numbers, with infection and cell number $Z$ scores of $-2.15$ and $+0.54$, respectively (Table S1). Four different siRNAs for Mib1 independently depleted Mib1 protein levels and reduced infection of HeLa cells, diploid human WI-38.
fibroblasts, and human epithelial lung carcinoma A549 cells with AdV-C5-E1A-FS2A-GFP (AdV-C5-GFP), whereas non-targeting control siRNAs (siNTs) had no effect (Figures 1C–1E). AdV-C5-GFP expressed stand-alone GFP from the immediate-early viral E1A-GFP hybrid mRNA with a “ribosome-skipping” F2A sequence (Minskaia et al., 2013), placed between the E1A and GFP open reading frames. We observed strong infection inhibition and loss of Mib1 in polyclonal CRISPR/Cas9 Mib1 knockout (KO) HeLa and A549 cells edited with a guide RNA directed to the first Mib1 exon but not a non-targeting control guide RNA (sgNT) (Figures S1C and S1D). The dramatic block of AdV-C5 infection was confirmed in a monoclonal HeLa-sgMib1 line (clone 1), which had an insertion of a thymidine at position 62 in all Mib1 alleles. HeLa-ATCC cells have three Mib1 genes (Adey et al., 2013). Insertion of a thymidine gave rise to a translational stop codon, TGA, 186 nucleotides downstream of the insertion and complete resistance to AdV-C5 infection, as indicated by lack of the immediate-early viral protein E1A (Figures S1E and S1F). Clone 1 was also resistant to AdV species A, B, and D but remained susceptible to herpes simplex virus 1 (HSV-1), pseudotyped lentivirus, vesicular stomatitis virus (VSV), and influenza A virus (IAV) infection (Figure 1F; Figure S1G). Mib1 KO cells even enhanced infection with the RNA viruses IAV and VSV, possibly because of reduced anti-viral activity in Mib1 KO cells (Li et al., 2011). Regardless, ectopic expression of Mib1 by a lentivirus in Mib1 KO cells completely restored AdV-C5 infection and Mib1 expression (Figures 1G and 1H).

**Loss of Mib1 Arrests Incoming AdV at the NPC and Blocks vDNA Uncoating**

AdV enters cells by receptor-mediated endocytosis, followed by penetration of the endosome, cytoplasmic transport, docking to the NPC, and release of the genome into the nucleus (Figure 2A; Greber and Flatt, 2019). Microscopic analyses of
single virions in single cells showed no reduction in Adv-C5 binding or endocytosis into cells treated with Mib1 siRNA or siNT and no exposure of membrane lytic protein VI or nuclear targeting (Figures 2B–2E; Figures S2A–S2D). Remarkably, sgMib1 cells appeared to contain more virions at the nucleus 5 and 8 h pi, suggesting that Mib1 was involved in turning over intracellular AdV particles. During AdV uncoating in unperturbed cells, AdV capsid fragments are displaced from the NPC to the cell periphery (Strunze et al., 2011). Electron microscopy up to 7 h pi indicated no reduction of incoming AdV-C5 at the nuclear membrane in sgMib1 cells, unlike control cells, which showed a strong decline in virions compared with 1 h pi (Figures 3A and 3B). This suggested a stable interaction of virions with NPCs in the absence of Mib1. This notion was supported by the finding that, in Mib1-depleted cells, more virions colocalized with NPCs 8 h pi than in control cells, as indicated by single-section super-resolution stimulated emission depletion (STED) microscopy (Figures S3A–S3C). It is of note that several capsid puncta did not perfectly colocalize with the anti-NPC antibody Mab414, which reacts with the central transporter protein p62, cytoplasmic filaments, and nuclear basket structures (Walther et al., 2001). Imperfect colocalization of AdV with Mab414 may reflect a heterogeneity of virion docking at NPCs (Cassany et al., 2015; Strunze et al., 2011; Trotman et al., 2001) or local curvature of the nuclear membrane.
The latter gives rise to lateral rather than “on face” views of AdV-NPC contacts, a phenomenon observed in the superresolved confocal images of the nuclear edge (Figure S3A).

Next we probed the genome contents of these particles using click chemistry, N3-Alexa Fluor 488, and confocal microscopy. Analyses of thousands of 5-ethynyl-2′-deoxycytidine (EdC)-labeled viral genomes confirmed the virtual absence of vDNA separation from the capsids up to 5.5 h pi (Figure 3C). Maximum image projections showed that nearly all virions of control cells released their genome either into the nucleus or misdelivered it to the cytosol (Figure 3D). Mib1-depleted cells, however, showed almost no free viral genomes in the nucleus or the cytoplasm but contained abundant amounts of EdC genome-positive virions, stained by the azide-conjugated fluorophore probe and contained in the leaky capsid. Such capsids are partly dismantled particles (Wang et al., 2013) and were readily detected in the cytosol of control cells and cells lacking Mib1 30 min pi (Figures 3C and 3D). Our analyses of single confocal mid-sections showed strong colocalization of viral DNA with the capsids at the nuclear membrane of sgMib1 cells and no evidence of vDNA in the nucleus, unlike control cells showing capsid-free viral genomes in the nucleus, as shown by lamin staining (Figure S3D). The data demonstrate that release of vDNA from capsids and vDNA nuclear import are dramatically impaired in the absence of Mib1.

Figure 4. Mib1 Ubiquitination Activity Is Required for AdV Genome Release

(A) Schematic representation of Mib1 domains.
(B) HeLa-sgMib1 cells were transduced with a lentivirus expressing the indicated constructs. Non-transduced HeLa-sgNT cells were included as a control. 48 h after transduction, cells were infected with AdV-CS-GFP. 24 h pi, cells were fixed, and nuclei were stained with DAPI before imaging by fluorescence microscopy. Infection was scored by measuring GFP intensity over nuclei. Error bars depict the mean ± SD of three replicates in one representative of three independent experiments. Statistical significance was assessed by one-way ANOVA with Holm-Sidak correction for multiple comparisons. ***p < 0.0001.
(C) HeLa-sgMib1 cells were transduced as in (B) and subsequently lysed and processed for SDS-PAGE and western blotting. Expression of FLAG-Mib1 mutants was probed with an anti-FLAG antibody, and FLAG-Cas9 served as a loading control.
(D) HeLa-sgMib1 cells carrying a tetracycline-inducible GFP-Mib1 cassette were incubated for 1 h with AdV-CS-EdC. Unbound virus was washed away, and internalized virus capsids were allowed to reach the nucleus for another 2 h. GFP-Mib1 expression was subsequently induced with 1 μg/mL doxycycline for 1 or 3 h. Then cells were fixed; stained for vDNA, hexon, and nuclei; and imaged via confocal fluorescence microscopy. Images are maximum projections. Three independent experiments gave similar results. Scale bar, 10 μm. See also Figure S4.
(E) Schematic model depicting the block in AdV DNA uncoating at the nuclear pore complex (NPC) in the absence of Mib1. Induced expression of the E3 ubiquitin (Ub) ligase Mib1 licenses AdV uncoating and vDNA nuclear import and misdelivery and leads to infection.
The Ub Ligase Activity of Mib1 Is Required to Reverse the AdV Uncoating Defect

Mib1 is an E3 Ub ligase with three main domains: an N-terminal region comprising Mib-Herc2 domains with a ZZ zinc finger, a middle region containing nine ankyrin repeats, and a C-terminal region containing three RING domains (Figure 4A). Whereas the N-terminal region mediates protein-protein interactions (Mertz et al., 2015), the C-terminal RING domain has E3 Ub ligase activity. We expressed various FLAG-tagged truncation mutants as well as two point mutants of Mib1 in sgMib1 cells by lentiviral transduction. Although AdV-C5-GFP infection was completely restored by expression of full-length Mib1, none of the truncation mutants rescued the infection, suggesting that both the N-terminal and C-terminal domains are required for AdV infection (Figures 4B and 4C). Expression of a full-length serine 805 to alanine (S805A) mutant abrogating phosphorylation at this position (Berndt et al., 2011; Ossipova et al., 2009) gave good rescue of infection. This stood in contrast to the substitution of cysteine 995 by serine (C995S), which did not rescue infection. The C995S mutant lacks ubiquitination activity because of a disrupted structure of the third RING domain (Berndt et al., 2011; Itoh et al., 2003). Staining of incoming vDNA confirmed that the truncation mutant 222–1,006 and the C995S mutant failed to induce genome release from the particles, unlike full-length Mib1 (Figure S4).

Mib1 localizes to microtubules in centriolar satellites near the microtubule-organizing center (MTOC). AdV traffics on microtubules to the vicinity of the nuclear envelope where it detaches from microtubules and binds to NPCs (Strunze et al., 2005; Wang et al., 2017). To distinguish whether Mib1 acted on the capsid in transit from the MTOC to the NPC or directly at the NPC-docked capsid, we infected HeLa-sgMib1 cells containing tetracycline-inducible GFP-tagged Mib1. Upon virus internalization and arrest at the NPC, we added doxycycline to induce expression of GFP-Mib1 and analyzed the infected cells for vDNA uncoating. GFP-Mib1 expression was detectable as early as 1 h after induction and led to release of the vDNA from nearly all capsids within 3 h of doxycycline treatment (Figure 4D). In contrast, in cells that did not express GFP-Mib1, the vDNA remained confined inside the capsids.

This result suggests that Mib1 performs a ubiquitination reaction directly at the site where the capsids are docked at NPCs. This notion was supported by live-cell imaging of atto565-labeled AdV-C5 particles in GFP-Mib1-expressing HeLa-sgMib1 cells, demonstrating that, as early as 60 min after doxycycline addition, Mib1 puncta overlapped with viral capsids in the nuclear vicinity (Video S1). The interactions of GFP-Mib1 with NPC-docked virions were transient, suggesting that Mib1 acts as a licensing factor on a cellular and/or a virion protein, leading to vDNA uncoating. Remarkably, the expression pattern and dynamics of Mib1-GFP in uninfected cells showed rapid, linear, and long-ranged trafficking of GFP puncta, indistinguishable from infected cells (Video S2). We speculate that the Mib1 ubiquitination activity primes a cellular and/or virion protein for dissociation from the virion and thereby unleashes the capsid disassembly reaction. Alternatively, ubiquitilation of a cellular inhibitor of the AdV uncoating reaction at the NPC could initiate release of a break in virion uncoating. Regardless, absence of Mib1 caused a very strong and highly specific entry phenotype at the NPC, comparable perhaps with temperature-sensitive virus mutants, such as the endosomal escape-defective AdV-C2-TS1 (Imelli et al., 2009) or HSV-1 mutants defective in uncoating at the NPC (Huffman et al., 2017; Knipe et al., 1981). These AdV and HSV-1 mutants have pleiotropic defects in the virion because of lack of proteolytic maturation (Imelli et al., 2009; Jovasevic et al., 2008).

Mib1 is abundantly available in most organs (Schmidt et al., 2018) and also in professional antigen-presenting cells, such as macrophages, which are infected by AdV (Li et al., 2011; Stichling et al., 2018). Besides being involved in Notch/Delta signaling (Itoh et al., 2003), Mib1 is implicated in centriole biogenesis (Cajánek et al., 2015), cell migration (Mizoguchi et al., 2017), and ciliogenesis (Wang et al., 2016), underscoring an important role in development (Koo et al., 2005). Point mutations in Mib1 can manifest in an autosomal-dominant fashion and cause disease, such as left ventricular noncompaction, the third most common cardiomyopathy (Luxán et al., 2013). In differentiated somatic cells, however, Mib1 offers a novel antiviral host target; for example, in the lungs, liver, gastrointestinal tract, or eyes. Because Mib1 is highly conserved, with homology of more than 95% in most mammals and more than 91% in distant vertebrates such as zebrafish (Apweiler et al., 2004), it may also support AdV infection in species other than Homo sapiens. In sum, our study identifies an “on-demand” tunable mechanism for delivery of vDNA into the nucleus and misdelivery to the cytoplasm (Figure 4E). The ubiquitination activity of Mib1 licenses AdV uncoating at the NPC. Targeted perturbations of host functions can now be applied to explore the uncoating mechanism of vDNA.

STAR Methods

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.11.064.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.B. and U.F.G.; Supervision, Project Administration, and Funding Acquisition, U.F.G.; Formal Analysis and Validation, Software, Data Curation, Visualization, Design and Performance of Cell-Based Experiments, M.B.; Electron Microscopy Experiments, M.B., J.W.F., and K.B.; Genome-wide siRNA Screen, B.C., D.S., and M.E.; Generation of Viruses, M.B., M.S., and S.H.; Data Analysis and Interpretation, M.B., M.S., J.W.F., and U.F.G.; Writing, M.B. and U.F.G.; Editing, M.B., M.S., J.W.F., and U.F.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-Adenovirus protein VI | Burckhardt et al., 2011 | N/A |
| Anti-Adenovirus antibody (anti-Hexon), Dil: 1:1000 | Millipore | Cat# MAB8052, RRID: AB_95243, |
| Mouse anti-Hexon antibody 9C12 | Varghese et al., 2004 | N/A |
| Mouse anti-E1A antibody M73, Dil: 1:500 | Millipore | Cat# 05-599, RRID: AB_309833 |
| Rabbit monoclonal anti-Mib1 antibody, Dil: 1:1000 | Abcam | Cat# ab124929, RRID: AB_11127834 |
| Rabbit anti-Mib1 antibody, Dil: 1:150 (IF) | Sigma | Cat# M5948, RRID: AB_1841007 |
| Mouse anti-GAPDH antibody, Dil: 1:2500 | Thermo Fisher Scientific | Cat# MA5-15738, RRID: AB_10977387 |
| Mouse monoclonal anti-flag antibody FG4R, Dil: 1:1000 | Thermo Fisher Scientific | Cat# MA5-91878, RRID: AB_1957945 |
| Rabbit polyclonal anti-flag antibody, Dil: 1:1000 | Sigma-Aldrich | Cat# F7425, RRID: AB_439687 |
| Mouse monoclonal anti-IAV NP antibody HB-65 | ATCC (Yamauchi et al., 2011) | Cat# HB-65, RRID: CVCL_4524 |
| Mouse monoclonal Mab414, Dil: 1:500 | Abcam | Cat# ab24609, RRID: AB_448181 |
| Goat anti-Mouse IgG antibody, AlexaFluor 488, Dil: 1:500 | Thermo Fisher Scientific | Cat# A-11029, RRID: AB_138404 |
| Goat anti-Mouse IgG antibody, AlexaFluor 594, Dil: 1:500 | Thermo Fisher Scientific | Cat# A-11005, RRID: AB_141372 |
| Goat anti-Mouse IgG antibody, AlexaFluor 680, Dil: 1:500 | Thermo Fisher Scientific | Cat# A-21058, RRID: AB_2535724 |
| Goat anti-Rabbit IgG antibody, AlexaFluor 488, Dil: 1:500 | Thermo Fisher Scientific | Cat# A-11034, RRID: AB_2576217 |
| Goat anti-Rabbit IgG antibody, AlexaFluor 594, Dil: 1:500 | Thermo Fisher Scientific | Cat# A-11037, RRID: AB_2534095 |
| Goat anti-Rabbit IgG antibody, AlexaFluor 680, Dil: 1:500 | Thermo Fisher Scientific | Cat# A-21109, RRID: AB_2535758 |
| Goat anti-Mouse IgG antibody, HRP-linked, Dil: 1:5000 | Cell Signaling | Cat# 7076, RRID: AB_330924 |
| Goat anti-Rabbit IgG antibody, HRP-linked, Dil: 1:5000 | Cell Signaling | Cat# 7074, RRID: AB_2099233 |
| Goat anti-mouse IgG antibody, Abberior STAR 635P, Dil: 1:100 | Abberior | Cat# ST635P-1001-500UG |
| **Bacterial and Virus Strains** |        |            |
| Competent cells DH5α strain | Hearing and Shenk, 1983 | N/A |
| AdV-C5 (wt300) | This paper | N/A |
| AdV-C5-GFP | | N/A |
| AdV-C5-EdC (genome-labeled) | Wang et al., 2013 | N/A |
| AdV-C5-atto565 (capsid-labeled) | Luisoni et al., 2015 | N/A |
| AdV-C5-Alexa Fluor 488 (capsid-labeled) | Burckhardt et al., 2011 | N/A |
| AdV-C2-ΔE3B-CMV-GFP | Yakimovich et al., 2012 | N/A |
| AdV-A31 | Anja Ehrhardt | N/A |
| AdV-B3 | Thomas Adrian | N/A |
| AdV-D8 | Anja Ehrhardt | N/A |
| Influenza A virus x31 (H3N2) | Virapur (Yamauchi et al., 2011) | N/A |
| HSV-1-GFP C12 | Glauser et al., 2010 | N/A |
| VSV-GFP | Hayer et al., 2010 | N/A |

(Continued on next page)
**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| DAPI (4',6-diamidino-2-phenylindole) | Sigma-Aldrich | Cat# D9542 |
| Lipofectamine RNAiMAX | Thermo Fisher Scientific | Cat# 13778075 |
| Alexa Fluor 647 NHS Ester (Succinimidyl Ester) | Thermo Fisher Scientific | Cat# A37566 |
| Alexa Fluor 488 Azide, Triethylammonium Salt | Thermo Fisher Scientific | Cat# A10266 |
| Alexa Fluor 594 Azide, Triethylammonium Salt | Thermo Fisher Scientific | Cat# A10270 |
| THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine) | Sigma-Aldrich | Cat# 762342 |
| AG (Aminoguanidine hydrochloride) | Sigma-Aldrich | Cat# 396494 |
| Copper sulfate | Sigma-Aldrich | Cat# 61230 |
| (+)-Sodium L-ascorbate | Sigma-Aldrich | Cat# 11140 |
| Doxycycline | Clontech | Cat# 631311 |
| ECL Prime Western Blotting Detection Reagent | Amersham | Cat# RPN2232 |
| **Critical Commercial Assays** | | |
| Zyppy Plasmid Miniprep Kit | Zymo Research | Cat# D4036 |
| NucleoBond Xtra Midi Kit | Macherey Nagel | Cat# 740410.100 |
| **Experimental Models: Cell Lines** | | |
| HeLa | ATCC | CCL-2, RRID: CVCL_0030 |
| HEK293T | ATCC | CRL-3216, RRID: CVCL_0063 |
| A549 | ATCC | CCL-185, RRID: CVCL_0023 |
| WI-38 | ATCC | CCL-75, RRID: CVCL_0579 |
| **Oligonucleotides** | | |
| See Table S4 | | |
| **Recombinant DNA** | | |
| lentiCRISPR v2 | Sanjana et al., 2014 | Addgene #52961 |
| eSpCas9(1.1) | Slaymaker et al., 2016 | Addgene #71814 |
| Lenti-eCas9 | This paper | N/A |
| Lenti-eCas9-sgMib1 | This paper | N/A |
| Lenti-eCas9-sgNT | This paper | N/A |
| pLVX-IRESPuro | Clontech | Cat# 632183 |
| pLVX-EGFP | This paper | N/A |
| pLVX-Mib1_res | This paper | N/A |
| pLVX-flag-Mib1 | This paper | N/A |
| pLVX-flag-Mib1-C995S | This paper | N/A |
| pLVX-flag-Mib1-S805A | This paper | N/A |
| pLVX-flag-Mib1-1-221 | This paper | N/A |
| pLVX-flag-Mib1-1-818 | This paper | N/A |
| pLVX-flag-Mib1-222-1006 | This paper | N/A |
| pLVX-flag-Mib1-222-818 | This paper | N/A |
| pCW57.1 | David Root | Addgene #41393 |
| pLVX-tet-BSD | This paper | N/A |
| pEGFP-C1 | Clontech | Cat# 6084-1 |
| pEGFP-Mib1 | This paper | N/A |
| pLVX-tet-BSD-EGFP-Mib1 | This paper | N/A |
| pCMV-dR8.91-Gag-Pol | Jovan Pavlovic (Crameri et al., 2018) | N/A |
| pVSV-G | Clontech | Cat# 631530 |
| p3HA-hMib1 | Vanessa Redecke | Addgene #33317 |
| **Software and Algorithms** | | |

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LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Professor Urs Greber (urs.greber@imls.uzh.ch). All reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Virus construction and growth

All AdVs were grown in human A549 or HeLa-ATCC cells and purified over two cesium chloride gradients as previously described (Greber et al., 1993, 1996). AdV-CS (wt300) has been previously described (Hearing and Shenk, 1983). AdV-CS-E1A-FS2A-GFP was constructed by inserting an enhanced GFP (GFP) cassette at the end of the E1A gene along with a furin cleavage site (FS) and a ‘ribosome-skipping’ F2A sequence derived from foot and mouth disease virus (Minskaia et al., 2013; Pacesa et al., 2017; Robinson et al., 2009), leading to expression of stand-alone GFP under the E1A promoter. This is akin to a mouse AdV-1 expressing murine granulocyte-macrophage colony-stimulating factor and human AdV-B3 expressing a GFP reporter (Pacesa et al., 2017; Robinson et al., 2009). AdV-C2-ΔE3B-CMV-GFP has been previously described and contains a GFP cassette under a CMV promoter in place of the non-essential E3B gene region (Yakimovich et al., 2012). Genome-labeled AdV-C5-EdC was produced by growing the virus in A549 cells in the presence of 2.5 μM EdC (5-Ethynyl-2'-deoxycytidine, Jena Biosciences) as described (Wang et al., 2013). Capsid-labeled AdV-C5-atto565 and AdV-C5-AlexaFluor 488 were generated as previously described (Burckhardt et al., 2011; Greber et al., 1998). Genome-labeled AdV-C5-EdC was produced by growing the virus in A549 cells in the presence of 2.5 μM EdC (5-Ethynyl-2'-deoxycytidine, Jena Biosciences) as described (Wang et al., 2013). Capsid-labeled AdV-C5-atto565 and AdV-C5-AlexaFluor 488 were generated as previously described (Burckhardt et al., 2011; Greber et al., 1998). A GFP-expressing VSV-G pseudotyped lentivirus (LV-GFP) was constructed by cloning a GFP cassette into the multiple cloning site of the lentiviral vector pLVX-IRES-puro (Clontech). The lentivirus was then rescued as described below. GFP-expressing vesicular stomatitis virus (VSV-GFP) was kindly provided by Y. Yamauchi (University of Bristol) (Hayer et al., 2010). Influenza A virus strain X31 (H3N2) was purchased from Virapur. GFP-expressing HSV-1 strain C12 was kindly provided by S. Efstathiou (University of Cambridge) (Glauser et al., 2010).

Generation of lentiviral particles

4.5x10⁶ HEK293T cells were seeded on a 10 cm dish. The next day, the cells were transfected with 3.4 μg pVSV-G (Clontech), 6.5 μg pCMV-dR8.91-Gag-Pol (kindly provided by J. Pavlovic, University of Zurich), and 10 μg of lentiviral expression plasmid using the calcium phosphate method. The culture supernatant was changed to fresh medium the following morning, and the supernatant containing the lentiviral particles was harvested at 2 days post transfection and filtered through a 0.45 μm filter before storage at –80°C. For experiments, cells were transduced with the lentiviral vectors at a MOI of 0.5 and selected with 2 μg/ml puromycin or 10 μg/ml blasticidin.

Reagents

Anti-AdV pan-hexon (mouse, MAB8052) and anti-E1A (mouse, 05-599) were purchased from Millipore. Anti-Mib1 (rabbit, ab124929) and anti-NPC Mab414 (mouse, ab24609) were purchased from abcam. Anti-GAPDH (mouse, MA5-15738), anti-flag (mouse, MA5-91878), and all secondary antibodies used for immunofluorescence were purchased from Thermo Fisher Scientific. Polyclonal anti-flag (rabbit, F7425) and anti-Mib1 (rabbit, M5948) were purchased from Sigma-Aldrich. All HRP-conjugated secondary antibodies used for Western Blotting were purchased from Cell Signaling Technologies. Rabbit polyclonal anti-AdV protein VI has been previously characterized (Burckhardt et al., 2011). Mouse monoclonal anti-AdV hexon 9C12 antibody was produced from hybridoma cell lines (Varghese et al., 2004). Mouse monoclonal anti-IAV NP antibody (HB-65) was a kind gift from Y. Yamauchi (University of Bristol). Lipofectamine RNAiMAX (13778075), Alexa Fluor 647 NHS Ester (A37566), Alexa Fluor 488 Azide (A10266), and Alexa Fluor 594 Azide (A10270) were purchased from Thermo Fisher Scientific. THPTA (762342), aminoguanidine hydrochloride (396494), copper sulfate (61230), and (+)-sodium L-ascorbate (11140) were purchased from Sigma-Aldrich. Doxycycline (631311) was purchased from Clontech.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GraphPad Prism version 8.0 | GraphPad Software | https://www.graphpad.com/ |
| Fiji | (Schindelin et al., 2012) | https://fiji.sc/#download |
| Leica Application Suite LAS | Leica | https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ts/ |
| CellProfiler version 2.1.1 | (Carpenter et al., 2006) | https://cellprofiler.org/releases/ |
| KNIME version 2.12.2 | KNIME | https://www.knime.com/downloads |

Continued
METHOD DETAILS

Cell cultures
HEK293T, HeLa, A549, and WI-38 cells were maintained in Dulbecco’s Modified Eagle’s Medium (GIBCO). Medium was supplemented with non-essential amino acids (Thermo Fisher) and 10% fetal calf serum (GIBCO). During infection experiments, the medium was additionally supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown at 37 °C in a 5% CO₂ atmosphere for no longer than 20 passages.

Genome-wide siRNA screen
The RNA interference screen was performed as part of the InfectX consortium using a commercially available pooled genome-wide siRNA library from Dharmacon (Ramo et al., 2014). The screen was conducted in HeLa CCL-2 cells in a 384-well plate format in two biological replicates. In each experiment, 25 μl of RNAiMAX (Invitrogen)/DMEM (0.1 μl / 24.9 μl) mixture was added to each well of the screening plates containing 1.6 pmol siRNA diluted in 5 μl RNase-free ddH₂O, after which the plates were incubated at room temperature (RT) for 1 h. Seven hundred HeLa cells were added per well in a volume of 50 μl DMEM/10% FCS, resulting in a final concentration of 6.7% FCS. Plates were incubated at 37°C and 5% CO₂ for 72 h prior to infection. All liquid handling stages of infection, fixation, and immunofluorescence (IF) staining were performed on the automated pipetting system Well Mate (Thermo Scientific Matrix) and washer Hydrospeed (Tecan). Adv-C2-ΔE3B-CMV-GFP was added to the cells at a MOI of 0.1 in 10 μl of infection media (DMEM supplemented with L-glutamine, 10% FCS, 1% Pen/Strep, Invitrogen). The cells were incubated at 37°C for 16 h and then fixed by adding 21 μl of 16% PFA directly to the culture supernatant for 45 min at RT or long-term storage at 4°C. Cells were washed two times with PBS containing 25 mM NH₄Cl and permeabilized with 25 μl 0.1% Triton X-100 (Sigma-Aldrich). After two washes with PBS the samples were incubated at RT for 1 h with 25 μl PBS containing DAPI (1 μg/ml, Sigma-Aldrich) and DY-647-phalloidin (0.4 U/ml, Dyomics), washed three times with PBS and stored until imaging in 50 μl PBS supplemented with NaN₃. Microscopy was performed with Molecular Devices ImageXpress microscopes. Nine sites per well in a 3x3 grid were imaged with a 10x S Fluor objective with 0.45 numerical aperture (NA) in a 12-bit dynamic range. Infection was scored by quantifying the GFP intensity over segmented nuclei (based on DAPI staining) and cell bodies (based on actin staining) using CellProfiler. All data generated during the screen including raw and processed images were shared through the openBIS system (Bauch et al., 2011). For the identification of pro-viral candidates, genes known to be involved in transcription and translation (such as polymerases, transcription factors, ribosomal proteins) were excluded based on their gene ontology terms. siRNAs that led to a strong reduction of cell numbers in the screen were additionally excluded in the validation experiments.

String analysis
Gene Ontology (GO) enrichment analysis of the strongest 500 infection up- and downregulating hits was performed with the “cellular components enrichment” tool of the STRING database (https://string-db.org/cgi/input.pl). Statistical significance for enrichment by genome-wide false discovery rate (FDR) was calculated using the STRING database (Szklarczyk et al., 2017).

Generation of CRISPR/Cas9 KO cell lines
For the generation of CRISPR/Cas9 knockout cells, we used a modified plasmid that we termed Lenti-eCas9. This plasmid was generated by replacing the Cas9 cassette from the lentiCRISPR v2 plasmid (Addgene #52961) (Sanjana et al., 2014) with the high-fidelity eCas9 from the eSpCas9(1.1) plasmid (Addgene #71814) (Slaymaker et al., 2016) by restriction cloning using XbaI and BamHI restriction sites. The sequences used as gRNA templates were 5'-GGTTGGCGCTGCGTATGCG-3' for MiB1 and 5'-GACGGAGGCTAAGCGTCGCAA-3' for a non-targeting gRNA, where the (G) denotes a nucleotide added for robust transcription from the U6 promoter. The sequences were cloned into the BsmBI site according to the instructions of the Zhang lab (Sanjana et al., 2014). All plasmids were verified by Sanger sequencing. The plasmids were then used for the generation of lentiviral particles. HeLa and A549 cells were transduced with lentiviral supernatant. After two days, the cells were selected with 2 μg/ml puromycin until all non-transduced cells were dead (ca. 5 days). Surviving cells from wells in which at least 50% of the cells had died were then expanded and frozen down. HeLa-sgMiB1 cells were furthermore subcloned by limiting dilution. Unless otherwise stated, all experiments were performed with HeLa-sgMiB1 cells grown from a single clone (cl. 1).

For sequencing analysis of the genome-edited cells, HeLa-ATCC and HeLa-sgMiB1 cells were each grown in a 10 cm dish. When they reached 90% confluence, cells were lysed in lysis buffer (20 mM Tris pH 8.0, 20 mM NaCl, 4 mM EDTA, 1% SDS) and sheared through a 22G needle. Proteins were digested by incubation with 450 μg proteinase K for 2 h at 55 °C under agitation. RNA was digested by incubation with 200 μg RNase A for 1 h at 37 °C. DNA was then isolated through two repeated phenol-chloroform extractions with a phenol:chloroform:isoamyl alcohol solution at ratios of 25:24:1, pH 8, and precipitated by addition of 1/10 the volume of 3M potassium acetate pH 5.2 and 2 volumes of EtOH followed by incubation at –20°C overnight. After washing with 70% EtOH, DNA pellets were resuspended in TE buffer (pH 8) and used for PCR amplification of a 300 bp stretch surrounding the Cas9 cut site on the MiB1 ORF using GoTag polymerase (Promega) with the following two primers carrying an Xhol or an EcoRI restriction site at their 5’ ends: 5’-TTGCTCTCGAGGCTAGTAATCCCGCGAATAAC-3’ and 5’-AAAGGAGATTCTCCTCCATGATAACACTGTG-3’. The resulting PCR product was then gel-purified and ligated into an Xhol/EcoRI-digested pBlueScript vector, which was then transformed into DH5α bacteria. The following day, more than 25 colonies were picked, their plasmid DNA isolated, and the PCR insert
was sequenced by Sanger sequencing. Every colony from the sgMib1 sample showed the insertion of a single thymidine. To corroborate this result, we sent the PCR product for sequencing without prior transformation to analyze the amplified DNA from all Mib1 alleles. We analyzed the sequences using the TIDE web tool (https://tide.deskgen.com/), which allows quantitative assessment of genome editing by sequence trace decomposition (Brinkman et al., 2014). This analysis showed that 98.2% of reads from the HeLa-sgMib1 cells contained the insertion of a single T, confirming that all alleles carried the same frameshift mutation. This is not surprising as the inDelphi tool (https://indelphi.giffordlab.mit.edu) which predicts the outcome of CRISPR/Cas9-mediated gene editing based on the gRNA target sequence context yields a high probability (> 70% in human cancer cell lines) for a 1-bp insertion for this particular gRNA targeting site (Shen et al., 2018). A list of all used oligonucleotides is available in Table S4.

Construction of expression plasmids

For constitutive expression, the lentiviral vector pLVX-IRES-puro (Clontech) was used. The Mib1 sequence was amplified by PCR from p3HA-hMib1 (Addgene #33317) with 5′-TAAGCAGAAATCATGAGTAACTCCGGAATAAC-3′ and 5′-TGCTTAGCCCGGCGCACACAC-3′ and cloned into the multiple cloning site (EcoRl/Notl) of pLVX-IRES-puro. To prevent cleavage of the re-introduced Mib1 gene by the Cas9/sgMib1 complex, the gRNA targeting site of Mib1 was mutated without changing the amino acid sequence via site-directed mutagenesis with the Q5 site-directed mutagenesis kit (New England Biolabs) according to the manufacturer’s instructions with 5′-agtgtgtcagaGGCCGGAGTGAATTGC-3′ and 5′-cctggcccttcACCTCTACCGAGCAC-3′. For detection of the Mib1 variants, a flag-tag was introduced at the N terminus of Mib1 using site-directed mutagenesis with 5′-cgaataaagcttacagatcagaagggcgccgtcAGTAACCTCCGGAATAAC-3′ and 5′-atgtccatcatctttatatacgctggctttgagcATCTGATTCCGAAATAG-3′. The Mib1 point and truncation mutants were constructed by site-directed mutagenesis with the primers in Table S4.

For inducible expression, we first created a tetracycline-inducible lentiviral vector of the pLVX-IRES-puro backbone termed pLVX-tet-BSD which has been previously used (Roulin et al., 2018). pLVX-tet-BSD was constructed by replacing the CMV promoter, IRES, and puromycin resistance gene in pLVX-IRES-Puro with a tetracycline response element (TRE) followed by a multiple cloning site and a blasticidin (BSD), a P2A sequence, and a reverse Tet repressor (rTetR) under a puromycin resistance gene in pLVX-IRES-puro with a tetracycline response element (TRE) followed by a multiple cloning site and cloned into the multiple cloning site (EcoRl/Notl) of pLVX-IRES-puro. To prevent cleavage of the re-introduced Mib1 gene by the Cas9/sgMib1 complex, the gRNA targeting site of Mib1 was mutated without changing the amino acid sequence via site-directed mutagenesis with the primers in Table S4.

SDS-PAGE

Cells from a 24-well plate were washed with PBS, lysed in 200 μl SDS-PAGE lysis buffer (200 mM Tris pH 6.8, 10% glycerol, 5 mM EDTA, 0.02% bromphenol blue, 5% SDS, 50 mM DTT) and boiled for 5 min at 95°C. Lysates were then loaded onto a SDS-PAGE gel and transferred to a PVDF membrane (Amersham). After blocking with blocking solution (5% milk powder in 20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5), the membrane was incubated with primary and secondary antibodies diluted in blocking solution at 4°C overnight or 1 h at RT, with four washes of TBST in between. HRP-coupled secondary antibody was detected using the ECL reagent (GE Healthcare). Primary antibodies were used at the following dilutions: anti-GAPDH 1:2500, anti-flag 1:1000, anti-GFP 1:1000, anti-AdV E1A M73 1:500. Secondary antibodies used in western blotting were goat anti-rabbit-HRP and goat anti-mouse-HRP used at 1:5000 dilution.

siRNA transfections

Two pmol siRNAs were diluted in a mixture of 9.85 μl OptiMem (GIBCO) and 0.15 μl RNAiMAX and spotted in a black 96-well plate (Greiner Bio-One). After 15 min incubation, 3000 cells were added per well in a volume of 90 μl DMEM supplemented with 10% FCS and nonessential amino acids (NEAA). The cells were typically incubated for 48 h before further treatment. The final siRNA concentration for all experiments was 20 nM. Unless stated otherwise, siMib1 #2 (Dharmacon) was used for depletion of Mib1 due to its strong knockdown efficiency.

Infection

Cells in a black 96-well imaging plate were ca. 50% confluent at the time of infection. The virus was diluted in infection medium (DMEM supplemented with 2% FCS, non-essential amino acids, pen/strep) to reach an infection of 20%–60%. The culture supernatant was aspirated and 100 μl of infection mix was added to the cells. For all AdV and lentiviral infections, the cells were fixed at 24 h pi with 4% PFA. For infections with HSV-1, VSV, and IAV, the cells were fixed at 7 h pi. After fixation, remaining PFA was quenched with 25 mM NH4Cl diluted in PBS for 5-10 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 3-5 min. If the virus carried a GFP transgene cassette, the cells were stained with DAPI at a concentration of 1 μg/ml in PBS for 3-5 min. For wild-type viruses, the cells were stained with the following primary antibodies diluted in blocking buffer (10% goat serum diluted in PBS) for 1 h at 4°C: mouse anti-hexon (Millipore, MABB052) or rabbit anti-protein VI (Burckhardt et al., 2011) for AdV, and mouse anti-NP (ATCC, HB-65) for IAV. After three washes of 4 min each in PBS, the cells were stained with secondary antibody (goat anti-rabbit-AlexaFluor 488 or goat anti-mouse-AlexaFluor 488, Thermo Fisher) diluted in blocking buffer containing 1 μg/ml DAPI for
30 min at RT. After three more washes of 4 min in PBS, the cells were imaged in a Molecular Devices high-throughput microscope (IXM-XL or IXMc) in widefield mode with a 20x objective. For quantification of infection with CellProfiler (Carpenter et al., 2006), the nuclei were segmented according to the DAPI signal, and the GFP intensity over the nuclear mask was measured.

Endocytosis and protein VI exposure
Forty thousand A549 cells were reverse-transfected with 20 nM siNT or siMib1 (Dharmacon) onto a coverslip in a 24-well plate. At 2 days post transfection, the cells were incubated with AlexaFluor 488-labeled AdV-C5 diluted in cold binding medium (HEPES-buffered RPMI supplemented with 0.2% bovine serum albumin) for 1 h on ice. Unbound virus was washed away three times with PBS, fresh medium was added, and cells were moved to a 37°C water bath to allow internalization of viral particles for 0, 10, or 20 min. Cells were then inverted onto a 30 µl droplet of 9Cl2 anti-hexon antibody (Varghese et al., 2004) diluted in cold binding medium and placed on ice for 1 h. The coverslips were then transferred back into the wells, washed 3x with PBS, and fixed with 4% PFA for 15 min at RT. The samples were then quenched with 25 mM NH4Cl for 5-10 min and permeabilized with 0.5% Triton X-100 in PBS for 3-5 min at RT. For immunostaining, the coverslips were inverted onto a 30 µl droplet of antibody diluted in blocking buffer (10% goat serum in PBS) containing an affinity-purified rabbit anti-protein VI antibody (Suomalainen et al., 2013) at 4°C for 1 h. After three washes of PBS for 4 min each, the cells were incubated with the appropriate secondary antibodies and the nuclei were stained with DAPI. The samples were imaged on a Leica SP8 confocal laser scanning microscope (cLSM). After recording the images, virus capsids were segmented using CellProfiler based on the AlexaFluor 488 signal. The signal from the hexon antibody determined whether the particle was outside or inside the plasma membrane. The signal from the protein VI antibody indicated successful exposure of protein VI, as the antibody cannot penetrate the virus capsids.

Virus binding
Forty thousand A549 cells were reverse-transfected with 20 nM siNT or siMib1 onto a coverslip in a 24-well plate. At 2 days post transfection, the cells were incubated with AdV-C5 diluted in cold binding medium on ice for 1 h. Subsequently, unbound virus was washed away with PBS and cells were fixed with 4% PFA. After fixation, remaining PFA was quenched and cells were permeabilized and stained with 9C12 anti-hexon antibody. Cell nuclei were stained with DAPI and cell outlines with AlexaFluor 647-conjugated succinimidyl ester. Cells were imaged on a Leica SP8 confocal microscope as described above. Nuclei were segmented based on the DAPI signal, cell outlines based on succinimidyl ester, and virus capsids based on the antibody signal.

Nuclear targeting
Eighty thousand A549-sgNT or A549-sgMib1 cells were seeded on coverslips in a 24-well plate. The following day, cells were incubated with AdV-C5 in cold binding medium for 1 h on ice. Unbound virus was washed away with PBS and cells were fixed with 4% PFA. After fixation, remaining PFA was quenched and cells were permeabilized and stained with 9C12 anti-hexon antibody. Cell nuclei were stained with DAPI and cell outlines with AlexaFluor 647-conjugated succinimidyl ester. Cells were imaged on a Leica SP8 confocal microscope as described above. For determination of nuclear targeting efficiency of virus, nuclei were segmented based on the DAPI signal, cells based on the succinimidyl ester signal, and capsids based on the antibody signal in maximum projection images. The number of capsids over the nuclear mask was then set in relation to the number of capsids over the entire cell.

Click chemistry and vDNA analysis
Cells grown on coverslips were incubated with genome-labeled AdV-C5-EdC (Wang et al., 2013) for various time points. After fixation, quenching, and permeabilization, samples were stained for incoming capsids with the 9C12 anti-hexon antibody. After primary and secondary antibody incubation, the coverslips were inverted onto a 30 µl droplet of click reaction mix for 2 h at RT. The freshly prepared click reaction mix consisted of 10 µM AlexaFluor 594- or AlexaFluor 488-conjugated azide (Thermo Fisher Scientific), 1 mM CuSO4, and 10 mM sodium ascorbate in the presence of 1 mM THPTA and 10 mM aminoguanidine (AG) in PBS. Samples were stained with DAPI and imaged with a Leica SP8 cLSM as described above. Single viral genomes and/or capsids were segmented according to the corresponding signal using CellProfiler.

Expression of Mib1 and Mib1 mutants
HeLa-sgMib1 cells were transduced with lentivirus containing expression cassettes of Mib1 or Mib1 mutants under a CMV promoter. Two days after transduction, the cells were infected with AdV-C5-GFP for 24h and the infection efficiency was quantified as described above. For the analysis of incoming vDNA, cells were incubated with AdV-C5-EdC for 1 h on ice followed by 3 h at 37°C. Cells were then processed for immunostaining and click chemistry. For analysis of Mib1 expression, cells were lysed in SDS-PAGE lysis buffer, and proteins were separated via SDS-PAGE, followed by western blotting with rabbit anti-flag antibody at 1:1000 dilution to detect the flag-Mib1 proteins.

Doxycycline-induced expression of Mib1
HeLa-sgMib1 cells which had been transduced with LVX-tet-BSG-GFP-Mib1 and selected for seven days with blasticidin were seeded on coverslips and infected with AdV-C5-EdC. After 1 h at 37°C, cells were washed and given fresh medium. The cells
were placed back at 37°C for an additional 2 h to give the virus capsids enough time to traffic to the nucleus. Subsequently, GFP-Mib1 expression was induced with 1 μg/ml doxycycline. After 1 or 3 h, cells were fixed and processed for immunostaining against hexon and click chemistry. Samples were imaged on a Leica SP8 cLSM.

Confocal microscopy

A Leica SP8 cLSM was used in all experiments, in which single viral particles and genomes were imaged. Imaging was performed at a scanning speed of 700 Hz with a 63x magnification oil objective with a numerical aperture of 1.40 and a zoom factor of 2, with a pixel size of 0.181 μm. z stacks were captured with a step size of 0.5 μm to capture the entire cell, and the size of the pinhole was 1 Airy unit. Sequential acquisition was between frames with line averaging (depending on the signal-to-noise ratio). Leica hybrid detectors (HyD) were used for each channel.

gSTED microscopy

Eighty thousand HeLa-sgMib1 cells were seeded on coverslips in a 24-well dish. The following day, cells were infected with 4 μg of AdV-C5-atto565. After 3 h, unbound virus was washed away and the cells were incubated in fresh medium for an additional 5 h. Cells were fixed, quenched, and permeabilized and subsequently stained with Mab414 antibody against nuclear pore complex (1:100 dilution) and goat anti-mouse-Abberior STAR 635P (1:100 dilution). The coverslips were mounted onto ProLong Gold Antifade Mountant (Thermo Fisher Scientific) and imaged the following day on a Leica SP8 inverse STED 3X microscope. Images were acquired with 100x, 1.40NA objective with a pixel size of 14 nm and gating from 1.5 to 6 ns. A 660 nm depletion laser was used for the atto565 fluorophore, a 775 nm depletion laser was used for Abberior STAR 635P. Due to slight drift during image acquisition, CellProfiler was used to align STED images from each channel to their respective confocal images, which were taken without STED mode at the beginning of the image sequence.

Confocal spinning-disc live microscopy

Eight thousand HeLa-sgMib1 cells transduced with a doxycycline-inducible GFP-Mib1 lentivirus were seeded in a 10-well CELLview slide (Greiner Bio-One) with a 175 μm thick cover glass embedded in its bottom. After two days, the cells were incubated with AdV-C5-atto565 for 30 min at 37°C, or left uninfected. Unbound virus was washed away and the cells were incubated for another 60 min at 37°C so that most viral capsids would reach the nuclear envelope. Fresh medium without phenol-red containing 1 μg/ml doxycycline was added to the cells to induce expression of GFP-Mib1, and live imaging was started 1 h later on a VisiGlu-W1 spinning disk microscope consisting of a Nikon Eclipse T1 microscope and a Yokogawa confocal scanning unit W1 with a stage top incubation system at 37°C and 5% CO2. Z stacks consisting of four steps with a step size of 1.4 μm were acquired every 30 s for ca. 120 min with a 100x oil objective (NA 1.4) and a pinhole of 50 μm. The focus was maintained with a perfect focus system (PFS). Sequential acquisition was between frames with line averaging (depending on the signal-to-noise ratio). Leica hybrid detectors (HyD) were used for each channel.

Transmission electron microscopy

2.5x10^6 HeLa-sgNT or HeLa-sgMib1 cells were seeded in 10 cm dishes. The next day, they were infected with 325 μg wt AdV-C5. After 1 h at 37°C, the virus inoculum was removed and the cells were fixed after 0, 1, 4, or 6 h of further incubation at 37°C. For fixation, cells were washed once with PBS-Ca2+/Mg2+ (PBS supplemented with 0.5 mM MgCl2 and 1 mM CaCl2) and then scraped off into 5 mL PBS-Ca2+/Mg2+. Cells were pelleted by centrifugation at 3000 x g for 5 min, yielding a cell pellet in solidified agar while the rest of the agar was discarded. The cell pellet was then incubated in a 1% reduced osmium tetroxide solution (freshly prepared from a 1:1 solution of 2% osmium tetroxide and 3% potassium ferricyanide) for 1 h on ice. Cells were then rinsed three times for 30 min in 750 mL PBS-Ca2+/Mg2+. Cells were then pelleted and resuspended in 400 μl 2% agar noble (BD Biosciences) solution in PBS-Ca2+/Mg2+ and placed at 65°C for 5 min. Cells were centrifuged in a swing rotor at 3000 xg for 5 min, yielding a cell pellet in solidified agar while the rest of the agar was discarded. The cell pellet was then incubated in a 1% reduced osmium tetroxide solution (freshly prepared from a 1:1 solution of 2% osmium tetroxide and 3% potassium ferricyanide) for 1 h on ice. Cells were then rinsed three times for 30 min in PBS-Ca2+/Mg2+ and 5 min in ddH2O, then pre-stained with a 2% uranyl acetate solution overnight at 4°C. For sample dehydration, the samples were successively incubated with 30% acetone for 5 min, 50% acetone for 5 min, 70% acetone for 30 min, 90% acetone for 10 min, 100% acetone for 5 min and 100% acetone for 10 min. Samples were embedded in a 48% epoxy resin containing 16% dodocenyl succinic anhydride, 34% methyl nadic anhydride, and 2% benzylidimethylamine. The resin was allowed to polymerize at 60°C for 3 days. Ultrathin 100 nm sections were obtained with a Leica Ultracut UCT ultramicrotome (Leica Microsystems) and mounted on copper grids with parlodion-carbon support film, placed sideways on a droplet of 2% uranyl acetate in H2O for 30 min, immersed repeatedly in H2O, dried overnight, placed on a droplet of Sato’s lead staining solution for 20 min, washed in H2O and then dried for 30 min. Samples were imaged in a FEI CM100 electron microscope at 80 kV.

Image analysis

Images were analyzed and quantified by using custom CellProfiler (version 2.1.1) and KNIME (version 2.12.2) pipelines. For infection assays, the nuclei were segmented using the DAPI channel, and the mean GFP intensity on the identified nuclei was measured. The
GFP intensity threshold for infection was based on the non-infected controls. For quantification of single viral particles and genomes, maximum projections of the image stacks were used. Nuclei and cell outlines were segmented according to the DAPI and the succinimidyl ester staining, respectively. In cases where no succinimidyl ester was included, the channel with the strongest cellular background was used for the cell segmentation. Viral particles and/or genomes inside the cell outlines were then segmented and the mean intensities of the other channels on these objects were reported.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Unless otherwise indicated, graphs display mean ± standard deviation (SD) and represent data from at least three independent experiments. All data were plotted and statistical analyses performed with GraphPad Prism 8.0 software (GraphPad). Significance indicated by asterisks is designated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, non-significant. The following statistical tests were used: Unpaired (two-tailed) t test, Figures 1E and 1F; Ordinary one-way ANOVA with Holm-Sidak correction for multiple comparisons, Figures 1C, 1G, and 4B; Mann-Whitney test, Figure 2B; non-parametric ANOVA (Kruskal-Wallis test) with Dunn’s correction for multiple comparisons, Figures 2C–2E, 3A, and 3C. As indicated in the relevant figures, the term ‘n’ refers to the number of cells (Figures 2B–2E and 3A; Figures S3B and S3C) or the number of viral particles (Figure 3C) used in the analysis.

**DATA AND CODE AVAILABILITY**

The data from the genome-wide RNAi screen are in Table S1. The results of the gene ontology enrichment analyses are in Tables S2 and S3. The code for the various scripts that were used to analyze the images are available by contacting the Lead Author.