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„The potential of CRISPR/Cas systems in treatment of Adenovirus infection“

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“We cannot fathom the marvellous complexity of an organic being; but on the hypothesis here advanced this complexity is much increased. Each living creature must be looked at as a microcosm - a little universe, formed of a host of self-propagating organisms, inconceivably minute and as numerous as the stars in heaven."

(Darwin, 1868)

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Abstract

Human adenoviruses are pathogenic non-enveloped linear double-stranded DNA viruses. In the majority of the population, the virus does not pose a relevant risk, as a competent immune system can successfully fight and clear up an infection. This usually goes hand in hand with a lifelong immunity. In immunocompromised patients or in closely packed environments however, infections pose a significant challenge. The risk of a disseminated disease, affecting the whole body, is high and so are reported mortality rates. Available treatment options are limited and unsatisfactory. Therefore, the search for new treatment options is a clear medical need. In recent years, RNAi and CRISPR/Cas based methods have increasingly moved into the spotlight as antiviral treatment platforms. While research is mostly pre-clinical, available clinical trial data highlights the process that has been made towards the patient.

In this thesis I investigated the application of CRISPR/Cas based methods and their combination with RNAi to treat adenovirus infection. Herein I first focus on the RNA targeting effector CasRx. We developed an optimized antiviral platform, delivered to cells by recombinant adenoviral vectors, which targets human adenovirus (HAdV-5) replication *in vitro.* In assays, inhibition rates of up to 2 orders of magnitude were achieved. In the second topic I explore different ways of combining RNAi and CRISPR/CasRx, generating multiple plasmids, each following a different approach of multiplexing. The two main options addressed are expression of artificial micro RNAs under the same promoter as the gRNA or together with CasRx. The data showed that using a processed direct repeat gRNA design seems to be the better choice in the former and a design supplementing lost stabilizing mRNA structures due to artificial micro RNA processing in the latter.

In the third topic I focus on DNA targeting Cas9. Similar to the former part, we developed an optimized antiviral system to target adenoviral replication *in vitro.* Here, inhibition rates of up 2 orders of magnitude using a single gRNA and up to three orders of magnitude, when using cidofovir in combination or a vector expressing four gRNAs, were achieved. It was also shown how to optimize cassette size by multiplexing gRNAs with artificial micro RNAs. Furthermore, sequencing of isolated HAdV-5 DNA from cells, transduced with the recombinant 4 times gRNA construct, showed mutations in more than 80% of viral genomes.

Summarized, these results demonstrate the promising potential of CRISPR/CasRx and CRISPR/Cas9, alone or in combination with RNAi or concurrent medication, against adenovirus infection and their application as means of antiviral defense in general.

Zusammenfassung

Humane Adenoviren sind pathogene unbehüllte lineare Doppelstrang-DNA-Viren. Für die Mehrheit der Bevölkerung stellt das Virus kein relevantes Risiko dar, da ein kompetentes Immunsystem eine Infektion erfolgreich bekämpfen kann. Dies geht in der Regel mit einer lebenslangen Immunität einher. Bei immungeschwächten Patienten oder in dicht besiedelten Gebieten, stellen Infektionen jedoch eine große Herausforderung dar. Das Risiko einer disseminierten Krankheit, die den ganzen Körper befällt, ist hoch, ebenso wie die gemeldeten Sterblichkeitsraten. Die verfügbaren Behandlungsmöglichkeiten sind begrenzt und unbefriedigend. Daher ist die Suche nach neuen Behandlungsmöglichkeiten eine klare medizinische Notwendigkeit. In den letzten Jahren sind RNAi- und CRISPR/Cas-basierte Methoden als antivirale Behandlungsplattformen zunehmend ins Rampenlicht gerückt. Während die Forschung zu einem großen Teil präklinischer Natur ist, zeigen die verfügbaren Daten aus klinischen Studien den Fortschritt auf dem Weg zum Patienten.

In dieser Arbeit habe ich die Anwendung von CRISPR/Cas-basierten Methoden und deren Kombination mit RNAi zur Behandlung von Adenovirus-Infektionen untersucht. Ich konzentriere mich zunächst auf den RNA-Targeting-Effektor CasRx. Wir haben eine optimierte antivirale Plattform entwickelt, die über rekombinante adenovirale Vektoren in Zellen eingebracht wird und dort die Replikation des humanen Adenovirus (HAdV-5) hemmt. In Assays wurden Inhibierungsraten von bis zu 2 Zehnerpotenzen erreicht. Im zweiten Thema untersuche ich verschiedene Möglichkeiten, RNAi und CRISPR/CasRx zu kombinieren, indem ich mehrere Plasmide generiere, die jeweils einen möglichen Weg des Multiplexings verfolgen. Die beiden primären Optionen sind die Expression von artificial micro RNAs unter demselben Promotor wie die gRNA oder aber zusammen mit CasRx. Die Daten zeigen, dass die Verwendung eines prozessierten gRNA-Designs im ersten Fall die bessere Wahl zu sein scheint und im zweiten Fall ein Design, das stabilisierenden mRNA-Strukturen ergänzt, welche durch RNAi Prozessierung verlorengegangen sind.

Im dritten Thema konzentriere ich mich auf das DNA-Targeting Enzym Cas9. Ähnlich wie im ersten Teil haben wir ein optimiertes antivirales System entwickelt, um die Replikation von Adenoviren *in vitro* zu hemmen. Hier werden Inhibierungsraten von bis zu zwei Zehnerpotenzen bei Verwendung einer einzelnen gRNA und bis zu drei Zehnerpotenzen bei Verwendung von Cidofovir in Kombination oder einem Vektor, der vier gRNAs exprimiert, erreicht. Es wird auch gezeigt, wie die Expressions-Kassettengröße durch Multiplexing mit artificial micro RNAs optimiert werden kann. Darüber hinaus wurde HAdV-5-DNA aus Zellen isoliert, welche mit dem rekombinanten 4-fach gRNA-Konstrukt

transduziert wurden. In mehr als 80% der viralen Genome wurden in der Sequenzierung Mutationen entdeckt.

Zusammengefasst zeigen diese Ergebnisse das vielversprechende Potenzial von CRISPR/CasRx und CRISPR/Cas9, allein oder in Kombination mit RNAi oder gleichzeitiger Medikation, gegen Adenovirus-Infektionen und das Potential als Mittel der antiviralen Abwehr im Allgemeinen.

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1 Introduction and scientific background

1.1 Adenovirus

1.1.1 Classification and Evolution

Human adenoviruses, like all adenoviruses, belong to the *Adenoviridae* family. *Adenoviridae* have been shown to infect a great variety of vertebrates, ranging from fish to mammals. They are classified into five genera: Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus and Ichtadenovirus. **Mastadenoviruses** infect mammalians, including dogs, bats, horses, humans, swine and mice. **Aviadenoviruses** infect birds and **Atadenoviruses**, named because of their bias of genomes with high A + T content, a broad range like reptiles, opossumns and birds. **Siadenoviurses** include viruses of birds, reptiles, and amphibians and **Ichtadenvoiruses** those of fish. Each genus comprises a range of different species, classified based on analysis of the hexon amino acid sequence, and types. Mastadenovirus, to which the human adenovirus belongs, contains the biggest group of known viruses, likely because of research focus and not necessarily because of higher diversity. While all Adenoviruses share a similar morphology, they differ in their genomic organisation, including size and protein composition. While individual adenoviruses are usually host species specific, it is possible for adenoviruses from different genera to infect a single species (Davison et al., 2003; Flint & Nemerow, 2017; Friedmann, 2016; Maclachlan & Dubovi, 2017).

Human Adenoviruses are classified into seven species A to G and various different types **Figure 1** (Ismail et al., 2018). Up to type 51, they were defined as serotypes, based on (i) neutralization of infectivity through targeting of epitopes on the hexon protein and (ii) hemagglutination-inhibition, directed against epitopes on the fiver protein (Crawford-Miksza & Schnurr, 1996; Dhingra et al., 2019). Newer types mostly relied on a genotype definition, which requires novel sequences or recombinant phylogeny in major capsid coding genes (Dhingra et al., 2019). Switching to “type”-nomenclature based on whole-genome sequences from serotypes reflected todays prevalence of genome sequence data usage. Through this duplication of names and conflicts in literature are prevented (Robinson et al., 2011). Most of the types belong to species HAdV-D, followed by HAdV-B. Recombination events are the main reason for the diversity of D species types. Adenovirus is considered to be a relatively

stable double-strand DNA (dsDNA) virus, with respect to point mutations and genetic drift. In the context of recombination events however, the genome is remarkably unstable (Ismail et al., 2018).

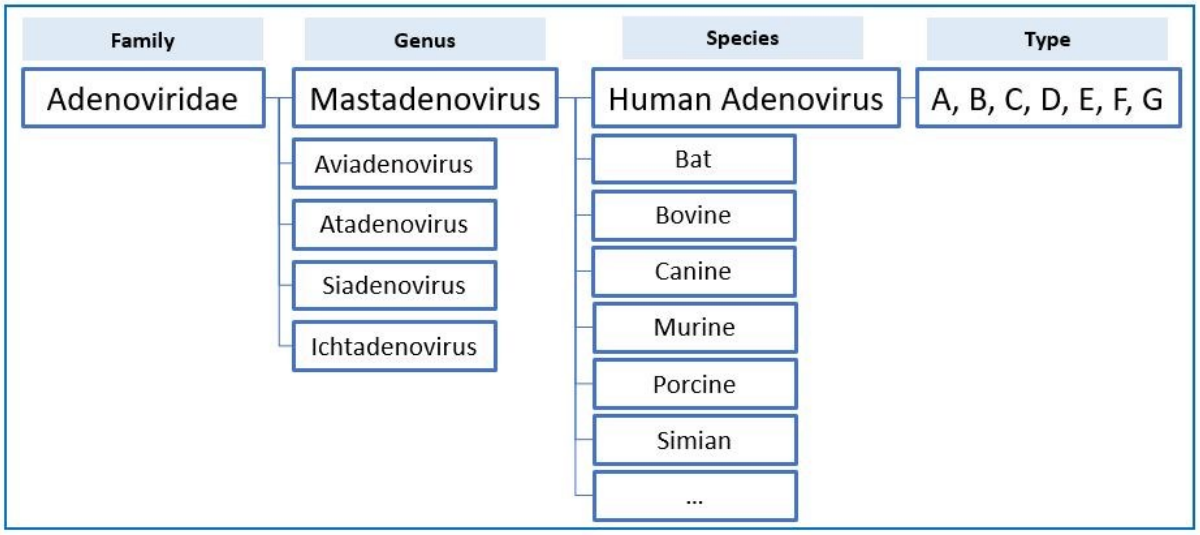


Figure 1 The *Adenoviridae* family, example of the human adenovirus. The *Adenoviridae* family consists of the genera Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus and Ichtadenovirus. The genera are further classified into species, such as human adenoviruses of the Mastadenoviruses, and types, A-G for human adenoviruses (ICTV, retrieved 2019).

Recombination in adenoviruses is a well-known factor and considered the main driver behind its evolution. In species D those events are mainly found in the surface proteins: penton, hexon and fiber. Homologous recombination seems to rely on regions with high sequence similarities within a species only and not across different species. However, non-homologous recombination in hypervariable regions of the hexon protein was also observed. Evolution of species D in particular seems to rely on this mechanism. Less so species B and only to a low percentage C (Ismail et al., 2018; Robinson et al., 2011). In species D, conserved sequence segments in the major capsid proteins facilitate the recombination events. In species C, having one of the lowest diversities in types and not harbouring such segments in its capsid proteins, recombination events in seem almost impossible. It is not completely clear on what mechanisms species C relies in terms of evolution. Recombination seems to play a role, although a menial one, and also immune escape mechanism are believed to be a factor, similar to what was previously described in species B (Dhingra et al., 2019).

1.1.2 Biology and Discovery

Human adenoviruses are pathogenic, non-enveloped DNA viruses with a linear double-stranded genome of 34-36 kb in size (Dhingra et al., 2019; Lynch et al., 2011). Their virion diameter ranges between 70 and 90 nm (Shakti Singh et al., 2019). They were first discovered 1952 by Rowe and Colleagues while investigating the growth of human adenoid tissue in culture. What they experienced was spontaneous degeneration due to a transmissible cytopathic agent. They called the new found pathogen "adenoid degeneration agent" (Rowe et al., 1953). The adenoids are lymphoid tissue in the pharynx. They play an important role in the immune defense against invading pathogens in the upper respiratory tract. Locally encountered antigens are inspected and destroyed. Effector and memory lymphocytes can then pass on the information to other areas in the body (Kempen et al., 2000). Since then, especially after it was demonstrated in 1960 that human adenovirus can cause tumours in experimental animals, they have been intensely characterized (Leppard, 2008; W. C. Russell, 2000). Human adenoviruses inherit a central role in biology and medicine. They were not only the first respiratory viral pathogens to be isolated and characterized but are also an important model organism. Fundamental discoveries in molecular and cellular biology, immunology, and systems biology are attributed to Adenovirus research. Examples include insights into messenger RNA splicing, eukaryotic DNA replication, and antigen presentation to T-Cells. Many more application areas have followed since (Ismail et al., 2018).

Adenovirus is of icosahedral structure, built by its major capsid proteins: hexon (protein II), penton (protein III) and fiber (protein IV). The hexon capsomere, formed as homo-trimers, compose the twenty triangular surfaces of the viral capsid, with penton capsomeres at its corners. Each hexon capsomere interacts with six other trimeres. The penton capsomeres are formed by five copies of penton base and three copies of fiber. They interact with the five hexon capsomeres at each corner of the adjacent surfaces. The protruding knobbed fibers facilitate entry of the viral particle into the host cell. Within the capsid, the core proteins V, VIII and μ are associated with the DNA. The latter being a processed form of polypeptide X. The Terminal protein sits at the ends of the dsDNA genome. Cement proteins further stabilise the capsid and make the connection between shell and core Figure 2 (C. S. Lee et al., 2017; Leppard, 2008; W. C. Russell, 2009).

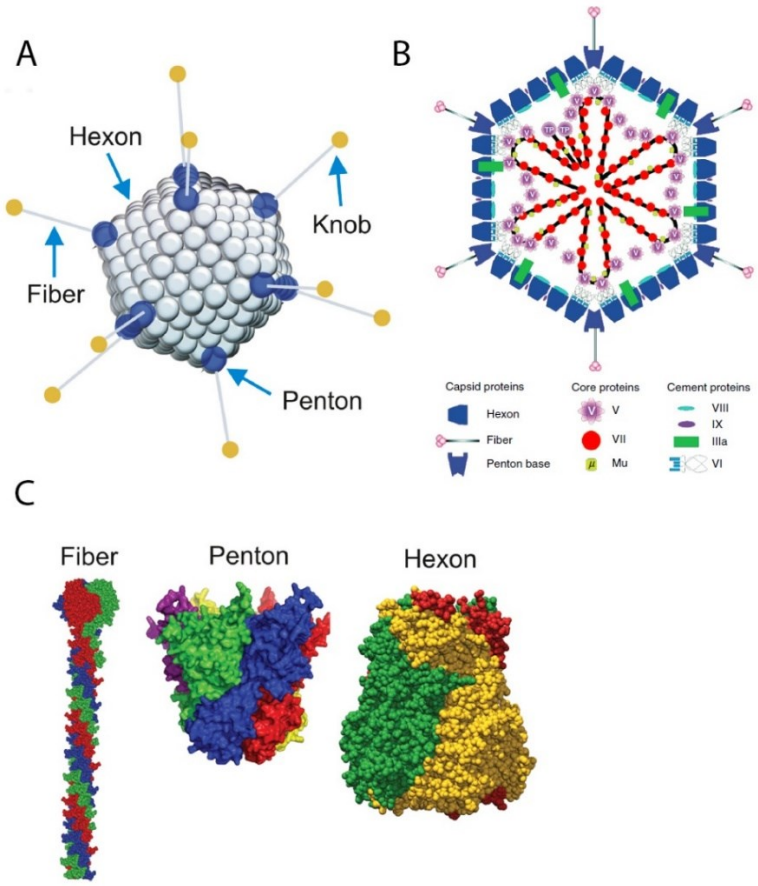


Figure 2 A: Icosahedral appearance of Adenovirus particle. Hexon capsomeres form the surface of the 20 triangular areas. Penton capsomeres with protruding knobbed fibres sit at the corner points. B: The core proteins are associated with the genome within the virus and the cement proteins further stabilise the capsid and bridge the gap between outer and inner shell, therefore forming a connection to the DNA. C: Crystal structures of the fiber, penton base, and hexon. The fiber shaft is formed by a homo trimer, as is the hexon capsomer. The penton capsomere is formed by five copies of penton base. Adapted from: (Flint & Nemerow, 2017; C. S. Lee et al., 2017; Leppard, 2008)

1.1.3 Genome Organisation

The linear dsDNA genome of adenovirus is transcribed from both strands. Overlapping open reading frames and alternative splicing further increase the coding potential and thus allow this rather small viral genome to express a multitude of proteins. It can be classified into immediate early and early genes, and in intermediate and late genes (Charman et al., 2019). Historically, expressed units were named solely based on their chronologically appearance during the infection cycle, E and L and ascending numbers: E1, E2, E3,... However, further investigations showed that this nomenclature is kept too general and does not reflect reality in enough detail. So, the newly described genes, within the transcription units, were given names that correspond to their expressed protein function (Flint &

Nemerow, 2017). The early transcription units, *E1-E4*, are expressed right upon entering the host cell. They have regulatory function, priming the host cell for viral replication, and are directly involved in viral DNA replication. The late units encode for structural proteins from which the viral capsid is formed (C. S. Lee et al., 2017). Additional functional elements are inverted terminal repeats (ITR) at the genome's ends, the packaging signal, virus associated RNAs I and II (VA) and the intermediate proteins IX and IVa2. The origin of replication is located at the terminal ends of each ITR and the packaging signal, required for virion assembly, proximal to the ITR. Virus associated RNAs interfere with the host cell immune defense and *IX* and *IVa2* promote transcription of the late phase genes and contribute to capsid formation

Figure 3 (Anderson et al., 2015; Charman et al., 2019).

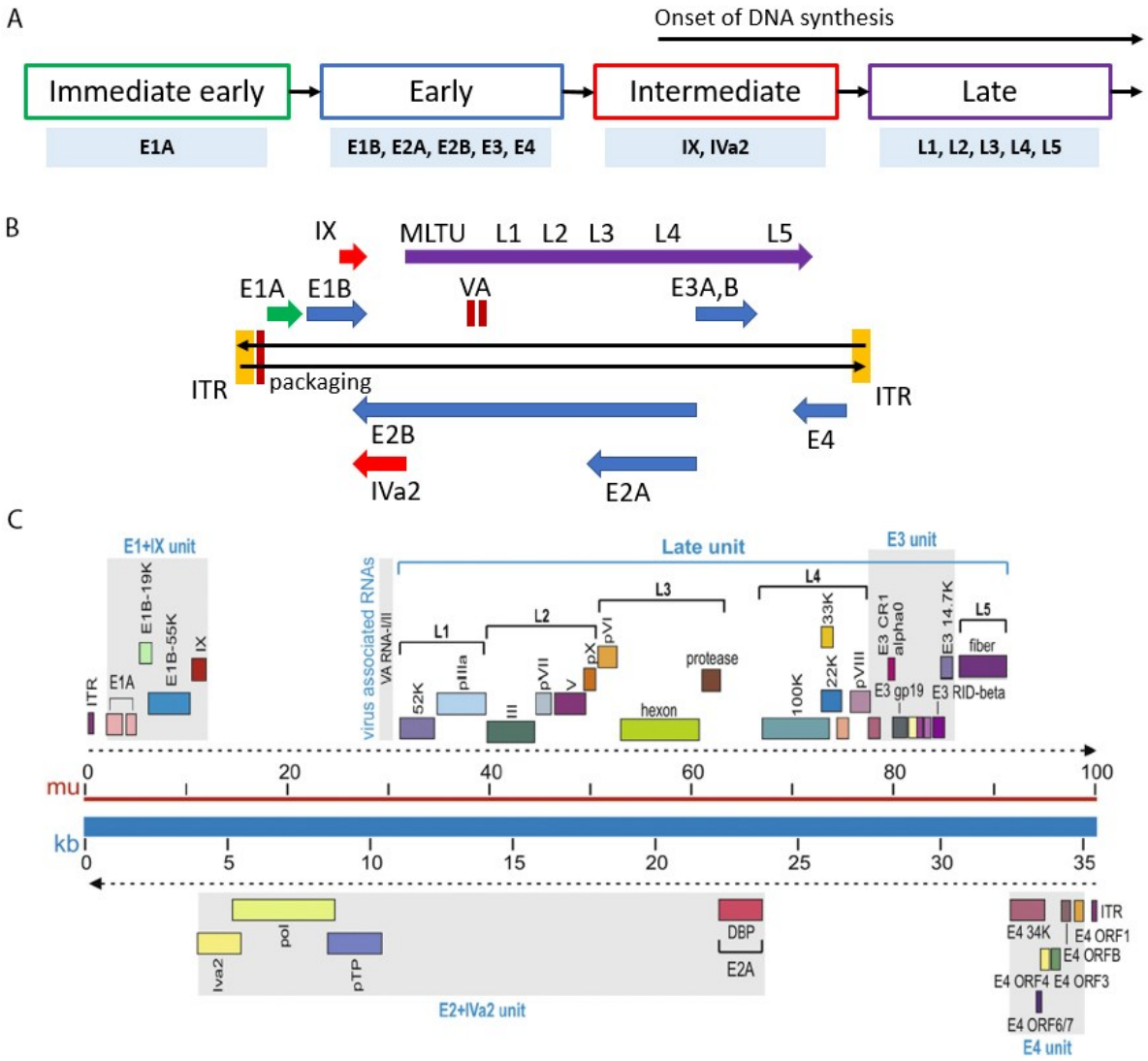


Figure 3 **Adenovirus genome organisation**. The adenovirus transcriptional units can be categorized by their expression timepoint during the infection cycle. Immediate early and early units are expressed first, being involved in priming the host cell's environment for viral replication, viral DNA synthesis and facilitating expression of the late phase genes. Late units code for structural proteins necessary to form the virions. The genome is transcribed from both ends and therefore a multitude of different encoded proteins are possible. Two inverted terminal repeats are located at the ends of the genome and proximal to one of the repeats lies the packaging signal. Marked in dark red are the virus associated RNAs (VA) that are involved in evading the host cell's immune defense. **A:** Classification of adenoviral genes in immediate early, early, intermediate, and late. **B:** Local organisation of genes, describing the major units and in more detail in **C.** A,B redrawn from: (Leppard, 2008) and C adapted from: (C. S. Lee et al., 2017).

1.1.4 Infection and replication cycle

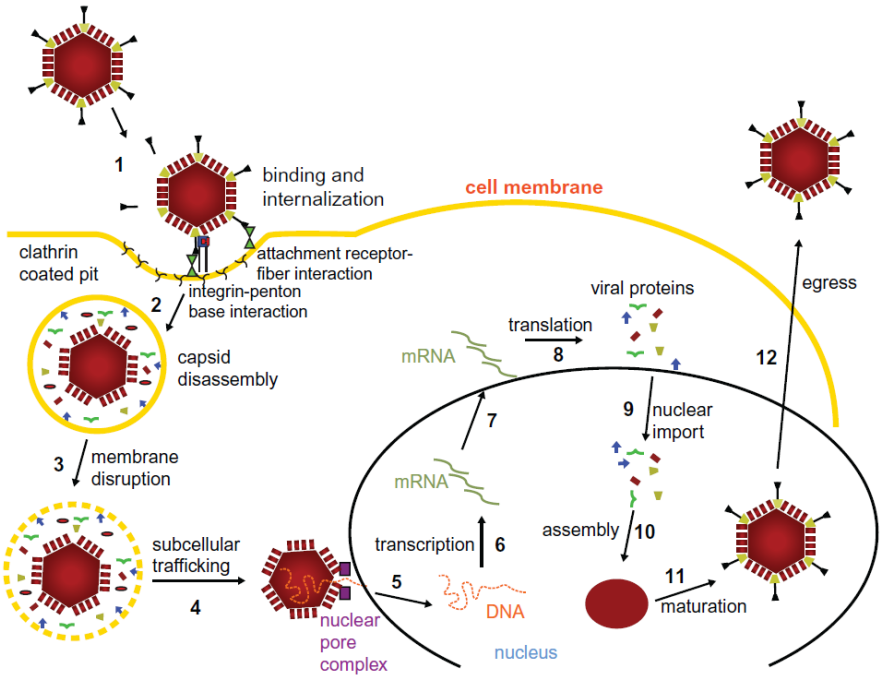


Figure 4 **Adenovirus infection and replication cycle. 1-2:** Attachment and entry is a two-factor process. Firstly, the virus' fiber protein binds to the primary high affinity receptor, secondly the penton base interacts with integrin. This leads to the formation of a clathirn coated pit and subsequently to an endosomal vesicle. **3:** The virus escapes the endosome, mainly through the help of protein VI and thus reaches the cytoplasm. **4:** The partly disassembled but still morphologically intact viral particle is transported along the microtubules to the nucleus where it connects to the nuclear pore complex. **5:** Being too large for simple internalization, the capsid dissembles and the genome with bound proteins is transported into the nucleus. **6-8**: The genome is immediately transcribed, and the mRNA, translated at the host cells' ribosomes. **9**: Produced viral proteins are transported into the nucleus due to their nuclear localization signals, or in case of hexon, mediated by pVI. **10**: The virion is assembled, and the genome packaged into it via the help of a packaging domain and packaging proteins. **11**: The adenovirus protease cleaves the viral precursor proteins to give them their final state. The thus matured viral particle is infections and primed for a subsequent host cell. **12**: Finally, the virus is released from the nucleus and the host cell altogether in lytic or non-lytic way, whereas lytic seems to be more efficient. Adapted from: (Flint & Nemerow, 2017) with information from: (Ahi & Mittal, 2016; Flint & Nemerow, 2017; Mangel & San Martin, 2014; Wodrich et al., 2003)

Attachment, entry and the interacting receptors

The first step in the adenovirus infection cycle is high affinity binding of the viral particle to the target cell. This bond is then followed by entry into the cell. Similar to other viruses, this is achieved by recognition of specific cell surface proteins and glycans. Studies, already in the early years of adenovirus research, have shown, that the interaction is mediated by the protruding fiber proteins. The host cell's counterpart remained elusive, however. Only a few years later the Coxsackie and adenovirus receptor (CAR), hence the name, was uncovered as responsible attachment partner for human adenovirus species C type 2 and 5. Since then a multitude of different attachment receptors have been identified. Some adenovirus types can also use more than one Figure 5 (Flint & Nemerow, 2017; Stasiak & Stehle, 2020).

Coxsackie and adenovirus receptor (CAR)

CAR is a transmembrane receptor, involved in cell-cell adhesion and highly expressed in epithelial cells. It can be found in many parts of the body, such as brain, heart, lung, liver, testis, pancreas, or kidney. Localized in tight junctions it plays a role in maintaining the integrity of the epithelium and helps in transmigration of leucocytes. Structural it consists of - starting C-terminal: a cytoplasmatic domain, transmembrane domain and two Ig-like domains, D2 and D1, extracellular (Ortiz-Zapater et al., 2017). Interaction of the adenoviral fiber happens via the N-terminal D1 domain (Zhang & Bergelson, 2005). When CAR is expressed on the basolateral surface of cells, like in the human airway system on polarized epithelial cells, the question arises how the virus can reach the receptors. There are a few possible considerations here: lesions of the epithelium that allow for viral entry, overproduction of fiber and therefore overflowing of the cells or attachment via apical expressed CAR isoforms. Apical expression of CAR can be enhanced by the host immune system, further increased through challenge with fiber protein and subsequent activation of inflammatory responses (Flint & Nemerow, 2017; Meier & Greber, 2004).

Human membrane cofactor protein 46 (CD46)

CD46 is a widely expressed transmembrane glycoprotein responsible for complement regulation. It can bind the complement proteins C3b and C4b and thus prevents the activation of the complement system that would otherwise be targeted against autologous cells. Unfortunately, like other complement proteins, it can also function as binding receptors for pathogens. Human adenoviruses

and other viruses such as measles and human herpesvirus 6 but also bacteria have been shown to interact (Gaggar et al., 2003; Stasiak & Stehle, 2020)

Glycans: GD1a

Glycans, a group of oligo- or polysaccharides, can be found on the surface of practically all the cells of all species examined so far. They comprise a large array of different functions, ranging from highly specific interactions to relatively non-specific effects. Usually, they influence a cell's response to environmental cues through linkage to other proteins or lipids. They have been around since the very early forms of cellular live (Varki, 2007). It is not unsurprising therefore that also pathogens have found ways to exploit them for their own good and use to use them for cell entry e.g. (Nilsson et al., 2011). The ganglioside, a sialylated glycan, GD1a is one of the most abundant gangliosides in the mammalian nervous system (Schnaar, 2016). During attachment the adenovirus fiber knob domain recognizes the two terminal sialic acids of Y-shaped GD1a (Nilsson et al., 2011).

Desmoglein-2 (DSG2)

Desmogleins belong to the cadherin cell adhesion molecule super family and are part of the desmosome (Hulpiau & van Roy, 2009). A desmosome is an intercellular junction found in tissues that have to withstand high mechanical stress like in muscles and skin e.g. They connect the cytoskeleton of the adjacent cells and therefore greatly improve the stability of the tissue. This connection is mediated by desmocollins and desmogleins, being inside of the junction between the cells (Delva et al., 2009; Harrison et al., 2016). In humans there are three desmocollin genes, *desmocollin1-3* and four desmoglein, *desmoglein1-4* (Dusek et al., 2007). Desmoglein-2 is composed of an intracellular domain that connects to the cell's cytoskeleton via plaque proteins, a single transmembrane domain, a cellular anchor domain (EA) and four extra cellular cadherin domains, EC1-EC4, N-terminal (Abrams & Saffitz, 2017). It has been shown that Desmoglein-2 can serve as entry receptor to human adenovirus. Receptor-virus binding takes place between EC2 and EC3, with EC3 being targeted first (Vassal-Stermann et al., 2019).

Integrins

Integrins, belonging to the cell adhesion receptors, can bind ligands on the extracellular matrix, on cell-surfaces and can also bind to soluble ligands. They are formed as heterodimers and consist of the subunits α and β. Both subunits can be present in different isoforms leading to at least 24 possible

heterodimers in humans. Both, α and β, have extracellular domains that contribute to the binding site. In general, an arginine-glycine-aspartic acid sequence serves as integrin-binding motif. However also motifs for specific proteins are known. Furthermore, they have transmembrane domains and intracellular domains. As soon as a ligand binds to the integrin receptor, a signal is generated intracellularly, which can influence the cell behaviour in various ways. Possible are interventions in proliferation, survival or apoptosis, adhesion, shape,… This is achieved mostly through the integrin's connection to the cytoskeleton. But the signal can also work in the other direction. For example, intracellular signals can influence the binding affinity of the extracellular domain of the receptor (Barczyk et al., 2010; Takada et al., 2007). During adenovirus infection integrins serve as coreceptors in virus internalization but not in initial binding, although there are cases reported where the integrin alone can function as receptor for infection (Nestic et al., 2019). After the virus has bound to its primary receptor, CAR e.g., the integrin receptor, consisting of the subunits αvβ1, αvβ3, αvβ5 e.g., subsequently binds the viral penton base. Through the following activation of signalling molecules, that lead to changes in the actin cytoskeleton, viral internalization is promoted (E. Li et al., 2001; Wickham et al., 1993).

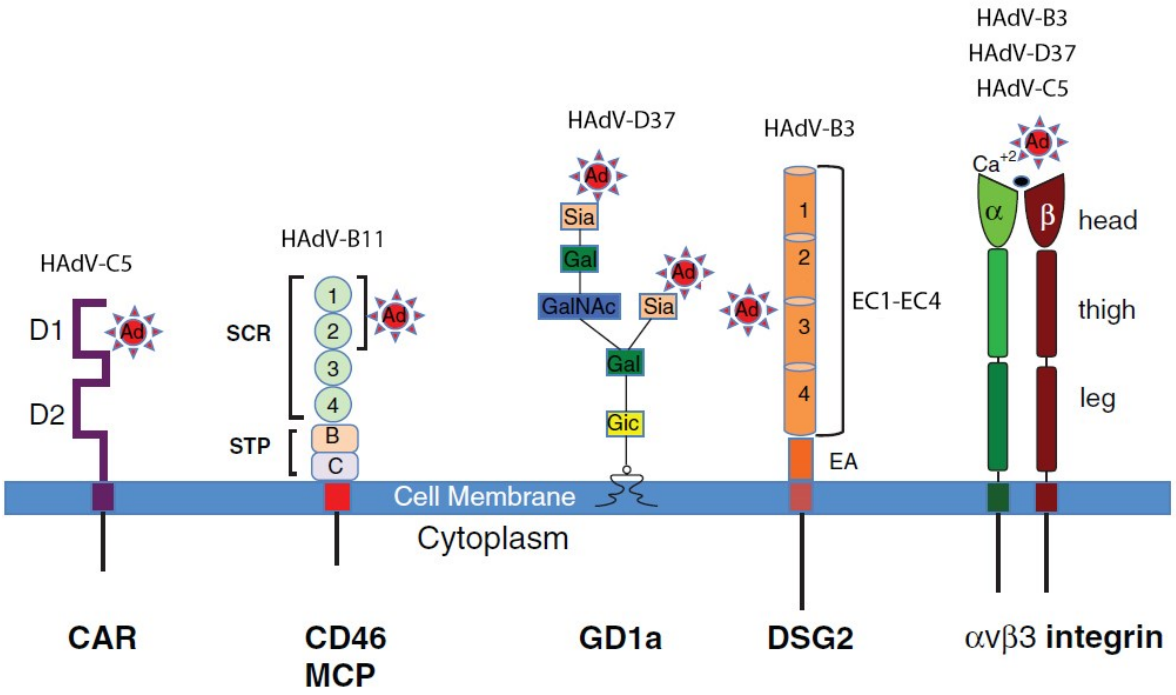
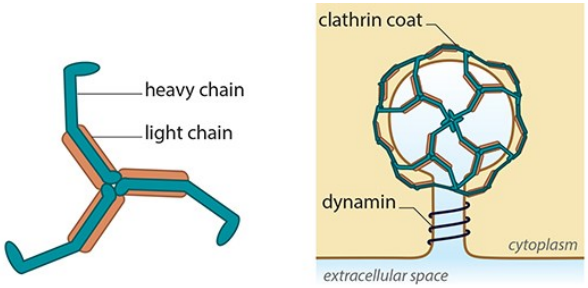


Figure 5 **Adenovirus receptors**. The first and one of the most important steps during the adenovirus infection cycle is high affinity binding to cell-surface receptors. A number of different receptors that can be used by the adenovirus have been identified. In this figure: coxsackie adenovirus receptor (CAR), human membrane cofactor protein 46 (CD46), the glycan GD1a, Desmoglein-2 (DSG-2) and integrin. Above the receptor schemata are example human adenovirus types that make use of the corresponding receptor. Internalization mostly relies on integrin as cofactor. The first bond to the primary receptor is mediated by the adenovirus' fiber, whereas the second interaction takes place between the penton base and integrin. Adapted from: (Flint & Nemerow, 2017).

As discussed above, after the connection between the virus' fiber protein and the primary receptor has been made, subsequent internalization mostly relies on interaction with an integrin coreceptor (Wickham et al., 1993). HAdV-5 first binds to CAR via the **fiber**, subsequently to integrin via the **penton** base and thus activates clathirn-mediated viral endocytosis. Clathrin, the key player in the endocytic pathway, forms a trimeric triskelion consisting of three heavy and light protein chain. Together with other triskelions, they coat the arising pit, built through clathirn polymerization and interaction with the actin cytoskeleton, in the cell membrane. The GTPase dynamin, forms a collar around the neck of the coated pit and facilitate detachment from the cell membrane. Clathrin completely engulfs the resulting vesicle only to be removed again in the next step, the uncoating **Figure 6**. Thereby the virus, within the now called endosome, has successful entered the cell (Kaksonen & Roux, 2018; Meier & Greber, 2004).



Clathrin triskelion Arising clathrin coated vesicle

Figure 6 **Clathrin organisation.** Clathrin is the main protein in the clathirn mediated endocytosis pathway. It consists of light and heavy chains that form a triskelion-like structure. Upon receptor-ligand binding, the cell membrane is deformed via the help of clathirn and interaction with the cytoskeleton. The thus arising pit is fully coated with clathrin triskelions. Dynamin and other proteins lead to subsequent scission from the cell membrane and the release of the finished vesicle into the cytosol. Adapted from: (MBINFO, retrieved 2022)

Endosomal escape, intracellular trafficking and entering of the nucleus

Although adenovirus has successfully entered the cell, the virion is still trapped in the endosome. For the next steps to take place, escape to the cytosol is necessary. Within the endosome, several proteins of the virion are dissociated, possibly due to acidification or other environmental clues, including fiber and **protein VI.** Protein VI is believed to cause the lysis of the endosomal membrane and therefore the release of the virus (Carrasco, 1994; Pied & Wodrich, 2019).

The virus particle now travels to the nucleus via microtubules. Interaction of **hexon** with the dynein motor protein in this context has been shown by (Bremner et al., 2009).

Finally at the nucleus, there is one last hurdle to overcome for the virus - the membrane of the nuclear envelope. The presence of enveloped organelles is a key characteristic of eukaryotic cells. Within the nucleus, enclosed by a double lipid layer, resides the genomic DNA. The valuable DNA thus benefits from additional protection and the cell is presented with another path of gene regulation. However, the DNA is not “cut-off” from the rest of the cell. Transcription factors and proteins involved in DNA transcription and replication pass inside and mRNA outside e.g. This exchange is mediated by the nuclear pore complex (NPC). Basically, it is a large number of specialized ducts, spread over the whole nuclear membrane. Macromolecules up to around 40kDa can pass through passively, whereas larger molecules require the help of transport factors. In contrast to most other membrane transports, the NPC can transfer molecules in their native folded state, enabling them to be active immediately after

transport. The size limit of passive diffusion is not to be understood as a definite threshold, but as a rapidly increasing energetic barrier, the bigger the molecule gets (Lin & Hoelz, 2019). Adenovirus reaches and binds the NPC as a still morphological intact particle, despite the loss of protein parts of its capsid. However, the icosahedral capsid exceeds the size limit of nuclear import. Therefore, the particle breaks up and the genome along with the attached core proteins, including TP, VII and μ is released for nuclear transduction (Flint & Nemerow, 2017; Pied & Wodrich, 2019).

Gene Expression

Immediately after the viral genome has entered the nucleus, the transcription of the same is started. For this purpose, the entire genome is read, as already described, in both directions. However, there are temporal differences in expression. It can be seen as a protein cascade, whereby the preceding gene products prepare for, or in case of the early genes, activate the following ones' expression (Leppard, 2008).

Virus associated non-coding RNAs

The first transcripts that are detected are the virus associated non-coding RNAs (VA). They are expressed by the host RNA Polymerase III, all other viral genes rely on transcription by host RNA Polymerase II (Crisostomo et al., 2019). VA RNAs were the first viral non-coding RNAs to be discovered. In around 80% of adenovirus representatives there are two of these: VA-I and VA-II. They are – especially in late phase of the infection - highly abundant double-stranded RNAs that can influence a variety of functions within the cell. Practical speaking, they interact with almost every mechanism that has something to do with dsRNA processing. The impact on the cellular RNA interference (RNAi) system and above all on protein kinase-R (PKR) is best described. PKR is a central part of the interferon pathway and as such, a major factor of the antiviral defense of the host cell. It is activated by dsRNA, from viral replication e.g. However, viruses have evolved ways to escape, such as through the VAs. Adenoviruses seem to mostly rely on VA-I, which inhibits PKR and thus prevents activation of the interferon pathway. The inhibition of PKR takes place via the steric structure of the VA-I. It was shown that longer linear RNA regions increase the likelihood of PKR activation. In addition, PKR itself is able to straighten flexible regions. The central domain of VA-I may specifically counteract on this factor. Furthermore, it may block the attachment of another PKR molecule and thus prevent

activation via dimerization or it may simply block the kinase domain required for activation (Hood et al., 2019).

VA-II, having arisen through gene duplication of VA-I, influences the cells' RNAi machinery (Hood et al., 2019). The VA RNAs not only have inherited function but are also incorporation into the cells' RNAi system, where they are processed into microRNAs. MicroRNAs (miRNAs) are small non-coding RNAs that influence gene expression in post-transcriptional manner. After transcription, they are not immediately functional. First the "pre-miRNAs" need to be transported to the cytoplasm using exportin-5 processed by dicer and incorporated into the RNA-induced silencing complex (RISC) **(see chapter 1.4).** In case of virus associated micro RNAs (mivaRNAs) they influence the cells' gene expression to support adenoviral replication (Wakabayashi et al., 2019). This is achieved either by sequence specific targeting of cellular mRNA, using the intended RNAi pathway or simply by competition for proteins, such as exportin-5, dicer and the RISC assembly. In the second case it is the high abundance and not the sequence of the mivaRNAs that conveys the effect (Piedade & Azevedo-Pereira, 2017).

The early gene set

The first of the larger genes expressed is ***E1A***. It is one of the most essential genes during the infection cycle. Initially activated by cellular transcription factors, E1A is able to further enhance its own expression (Crisostomo et al., 2019). Assays in which it was targeted by RNAi based strategies, impeded viral replication (Ibrisimovic et al., 2013). During splicing, multiple isoforms arise, whereas the two largest ones, encoding proteins of 289 and 243 amino acids are most abundant during the early infection phase. The gene's sequence contains 4 conserved regions (CR1-CR4), found across many human adenovirus species. It is believed that these regions offer an evolutionary advantage, explaining the conserved character. No known cellular orthologs exist, rather it is assumed that the functional motifs of E1A, developed in parallel. E1A prepares the host cell's environment for viral replication and pushes it into S-phase. Furthermore, it activates and modulates expression of the subsequent viral genes. The highly altered expression profile of infected cells underlines the enormous impact E1A exerts (King et al., 2018).

The next early genes in the cascade are **E1B**, **E2**, **E3** and **E4** (Leppard, 2008). **E1B**, consisting of a 55K and 19K protein, together with **E4 ORF6 (E 34K)** and **E4 ORF3**, inactivate the stress and DNA damage

sensor of the host cell - p53 (Dobner et al., 1996; Lomonosova et al., 2005; Soria et al., 2010; Turner et al., 2014). Furthermore, E4 ORF6 and E4 ORF3 interact with DNA-dependent protein kinase (DNA PK), preventing double-strand DNA break repair and thus undesired concatemer formation of the viral genome (Boyer et al., 1999). E4 ORF4 influences the host DNA damage response, viral splicing, and other factors concerning viral replication trough interplay with its main partner protein: protein phosphatase 2A (PP2A) (Kleinberger, 2020). E4 ORF1 changes the cellular glucose metabolism, leading to upregulation of glycolysis and thus promotion of nucleotide biosynthesis from glucose intermediates, further supporting viral replication (Thai et al., 2014). E4 ORF2 (E4 ORFb) is active during the lytic cycle (Dix & Leppard, 1995) and E4 ORF6/7 binds to the host E2F transcription factor and subsequently to the *E2* promoter for activation (Yamano et al., 1999).

In the *E2* region the genes *pol*, *pTP* and *DBP* are expressed (Leppard, 2008). The viral DNA polymerase (*pol*), the precursor of the terminal protein (*pTP*) and the DNA binding protein (*DBP*) along with cellular transcription factors form the DNA replication preinitiation complex (Parker et al., 1998).

Like the *E4* region the *E3* consists of a manifold of encoded proteins. E3 gp19K inhibits the transport of the class I major histocompatibility complex (MHC) from the endoplasmic reticulum, where it is loaded with peptides to present, to the cell membrane. Furthermore, it inhibits the necessary peptide processing step by tapasin, thus decreasing the total number of presented peptides in the first place. E3 14,7K, E3 10,4/14,5K and E3 RIDα/β inhibit TNFα receptor induced cell apoptosis (Horwitz, 2004).

Replication of the genome and the viral replication compartments

The replication of the adenovirus genome makes use of a strand displacement mechanism. Three viral proteins are necessary: pTP, Pol and DBP. Together with the host transcription factors NF1 and Oct-1, it is called the pre-initiation complex. Association with host transcription factors further enhances and finally initiates the replication by changing the origin's conformation (Charman et al., 2019). The origin of replication is a sequence of 51 bp, located within the two inverted terminal repeats (ITRs) at the ends of the viral genome. Only the core region of 18bp seems to be essential for initiation. It is this region that binds the pre-initiation complex (Leppard, 2008). The adenoviral DNA polymerase *pol* uses a protein primer (*pTP*) for initiation, while also featuring a 3'-5' exonuclease domain N-terminal, thus allowing for proofreading activity. After the priming event the polymerase dissociates from pTP and

continues the strand synthesis. **pTP** stays attached to the DNA end and is processed by the viral protease in a later step, becoming the terminal protein (**TP**). Elongating one DNA strand displaces the other, which becomes a new template on its own. Being involved in different steps during the viral infection cycle, **DBP** carries out one of its most important functions during replication. **DBP** binds to the displaced strand, helps in unwinding the remaining portion and provides protection from nucleases. Single stranded DNA intermediates accumulate during replication, their replication seemingly lagging behind. Nevertheless, also the ssDNA intermediates are replicated. The **ITRs** anneal together, building a panhandle-like structure and thus forming a new dsDNA origin of replication **Figure 7** (Charman et al., 2019). In cases where the replication starts simultaneously from both ends, no panhandle like structure is necessary as both origins are still double-stranded at the time of initiation (Leppard, 2008).

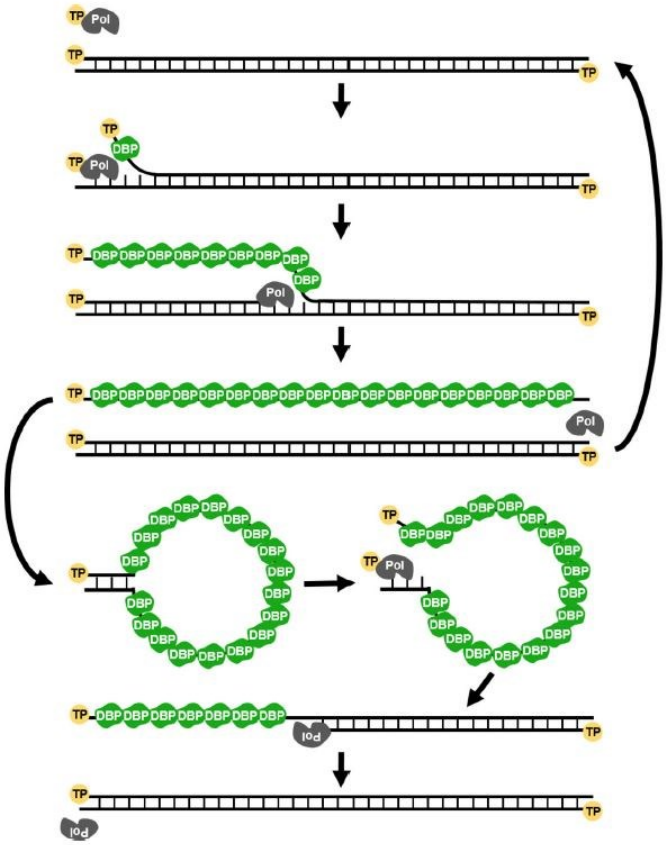


Figure 7 **Adenovirus DNA Replication**. Adenovirus DNA replication follows a strand displacement principle. The pre-initiation complex, consisting of pTP, Pol, DBP and host transcription factors bind the origin of replication, localized within the ITRs. The viral polymerase Pol uses pTP as primer and begins replication of one DNA strand. Simultaneously, with the help of DBP, the second strand gets displaced. DBP also binds to the arising single stranded DNA and thus provides protection from nucleases. The ssDNA now forms a panhandle like structure, end sitting ITRs annealing, to provide a new dsDNA origin of replication. DNA synthesis continues as before. Adapted from: (Charman et al., 2019).

Viral replication compartments and virus induced post replicative bodies

As mentioned before, all of this takes place in the host cells' nucleus. The nucleus is not only home to the DNA-encoded genetic information, but also a highly dynamic environment for an enormous number of cellular processes. For this, the nucleus is by no means an unorganized mixture of proteins and other molecules. One can find distinct subnuclear compartments of different functions. As with cell organelles, but without their own membrane, these nuclear bodies are places where various functions are carried out. The localized organization enables biological processes to run much more efficiently than with free floating components (Mao et al., 2011). Also adenovirus, and other DNA viruses, make use of this principle. Their replication takes place in so called viral replication compartments (VRCs). This gives protection to the viral genome from host cell defense mechanisms and, as is the case with nuclear bodies, provides a highly efficient environment for the virus' biosynthesis processes. More than one VRC is possible, whereas in HAdV the number corelates with the virus titer (Charman & Weitzman, 2020).

In recent years studies have tried to visualize VRCs. The results considered together, the replication centres seem to undergo maturation over time. Early during infection, distinct areas where ssDNA intermediates accumulate – the ssDNA accumulation sites (ssDAS) – are surrounded by areas of DNA replication – the peripheral replicative zones (PRZ). Later, foci of ssDNA enclose accumulations of replicated viral DNA of ever-increasing size. They are called virus induced post replicative (ViPR) bodies Figure 8 (Charman et al., 2019; Charman & Weitzman, 2020; Garces et al., 2016; Hidalgo & Gonzalez, 2019).

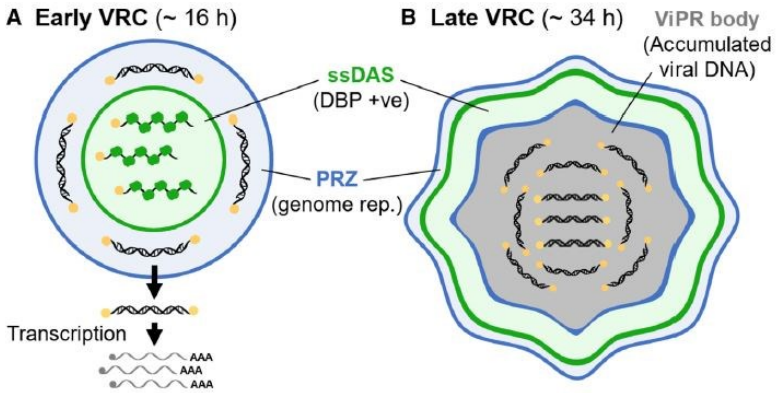


Figure 8 **Adenovirus replication compartment (VRC) and virus induced post replicative (ViPR) bodies**. The nucleus is a highly dynamic compartment of a cell. Biological processes are not occurring unorganized throughout the nuclear space, but rather happen in self-contained compartments. They are similar to cell organelles but don't possess any membrane. Compartmentalization is supporting efficient execution of cellular reactions. This also applies to adenoviral replication happening in so called VRCs. Over the course of the infection cycle, VRCs undergo maturation. **A**: in the early phase, spots of accumulated ssDNA intermediates, the ssDNA accumulation sites (ssDASs), are surrounded by areas of genome replication, the peripheral replicative zones (PRZs). Transcription takes place further outside. **B**: Later in the infection, spots of ssDNAs cluster around areas of accumulated replicated viral DNA of increasing size, the ViPR bodies. Adapted from: (Charman et al., 2019)

The **ViPR** bodies also include the viral packaging protein **IVa2** as well as the core protein **VII**. **PIX** and **pVI** are in close proximity (Genoveso et al., 2020). **IVa2** helps in packaging of the viral genome, takes part in the virion assembly, and activates the late transcription by applying its DNA binding motifs (Ostapchuk & Hearing, 2008). **VII** binds the viral DNA and may act in histone-like manner, condensing the DNA into nucleosome-like structures. Protection of the genome and assistance at the nuclear pore complex are further functions of **VII** (Inturi et al., 2013). **pIX** is best known for its structural role in the mature capsid. It sits in cavities between the Hexons and provides, non-essential although beneficial, additional stability to the virion. Despite its role as capsid protein, it is already expressed at an intermediate time during infection, with its levels greatly increasing after DNA replication has started. Whereas the protein's N-terminus is important for its role as capsid protein, the C-terminus shows transcriptional activity. As transcriptional activator **pIX** is able to influence viral gene expression of early and late regions. To what extent and importance, however, is a matter of debate (Parks, 2005).

Having areas of accumulated replicated viral DNA, together with packaging- and capsid proteins late in infection cycle, it is plausible that these can be seen as direct preparation of capsid assembly and maturation (Genoveso et al., 2020).

Capsid assembly, packaging, maturation, and release

Nuclear import of proteins

The viral capsid is assembled within the nucleus. The proteins required, are produced in the cytoplasm and must be transported inside first (Leppard, 2008). Normally, proteins are transported by nuclear localization signals (NLSs). These are short stretches with high rate of basic amino acid residues that are recognized by heterodimeric importin receptors at the nuclear pore complex. The dimers consist of an α protein that binds the NLS and a β protein that directs the complex to the nuclear pore. α is bound to β via the importin β binding domain (IBB). Interestingly, without β, NLS-like sequences of the alpha's IBB bind to its NLS-binding site and thus prevent, binding to NLS sequences on cargo proteins. Binding of β on the other hand, relies on the GTPase Ran, which at the same time, serves as environmental cue for the unidirectional transport into the nucleus. In the cytoplasm, Ran is found primarily bound to GDP, in the nucleus to GTP. Starting in the cytoplasmn, β binds to α, freeing up its NLS binding site. α binds to the cargo protein via the NLS-sequence. The complex, with the help of β, moves through the nuclear pore. Inside Ran-GTP binds to β, thus releasing α. The NLS-like domain of the alpha's IBB now competes with the bound NLS-signal, displacing the cargo protein because of higher affinity. The transport is complete (Hodel et al., 2001). α then moves back to the cytoplasmn via the help of a nuclear export factor and β, still bound to Ran-GTP, returns on its own. In the cytoplasmn Ran-GTP is hydrolized to Ran-GDP, thus released, freeing up β for the next transport cycle (Weis, 2003).

While most of adenoviral proteins rely on this mechanismn, the hexon protein does not posess NLS signals but relies on protein **pVI**. **pVI** binds to the Hexon via hydrophobic interactions at its nuclear export signal (NES). With NES thus shielded, unintended export of the Hexon bound complex right after import is prevented. This heterodimer subsequenlty recruits the importin α/β receptor via the NLS of **pVI** and the entire complex is moved into the nucleus where it dissacociates. **pVI** may continue in two ways. When cleaved by the viral **protease** (AVP) at its C-terminus, it looses the NLS, the NES is disrupted and thus the affinity for hexon increased. This happens predominatly in the late phase of infection, therefore priming the protein for virion assembly, where it bridges the gap between capsid and inner core within the mature virion. Remaining unprocessed on the other hand, it is able to return to the cytpoplasma, via bininding of the export complex to the NES, picking up the next Hexon **Figure 9** (Wodrich et al., 2003).

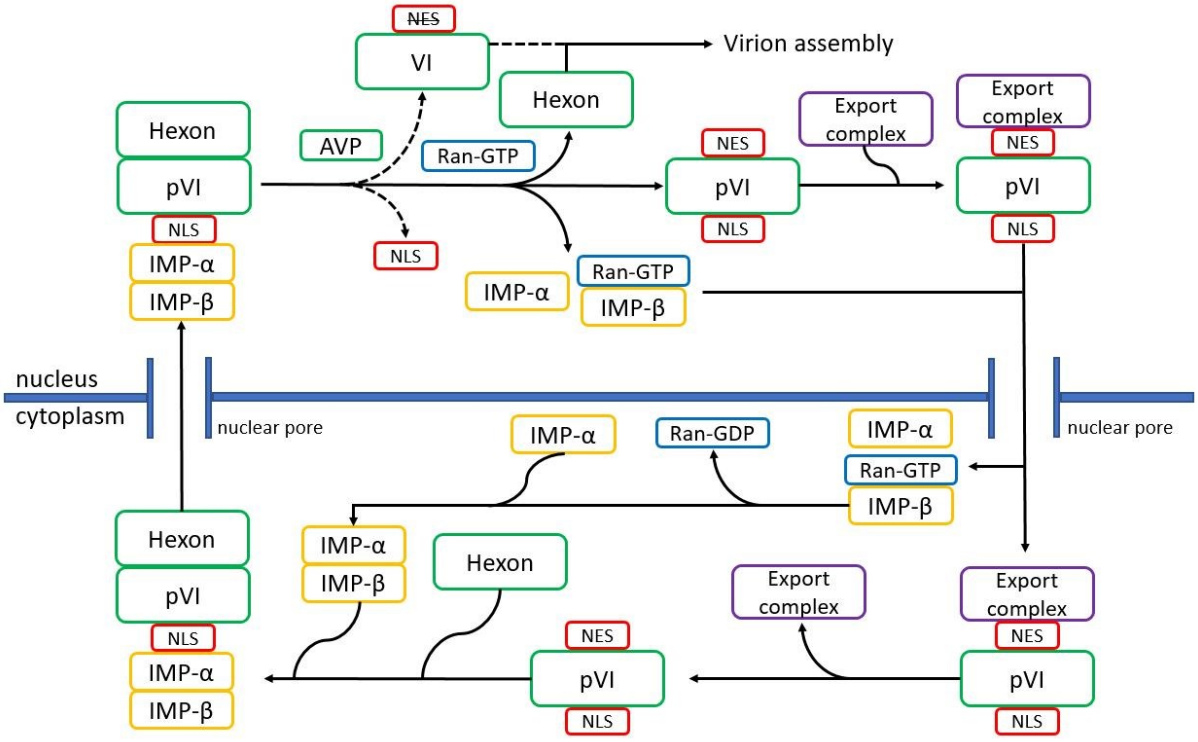


Figure 9 **Nuclear import of viral proteins**. Usually, proteins are imported into the nucleus via nuclear localization signals (NLS). Because of the cytoplasmatic environment being rich in Ran-GDP, β importin binds to α, freeing up its NLS binding domain. α subsequently binds to the NLS of the cargo protein and the complete comlex is moved into the nucleus, by the interaction of the β importin with the nuclear pore complex. Inside, Ran-GTP binds to β and the complex dissasociates. The protein has been sucessfully transported and α and β return to the cytoplasma. α with the help of an export protein, β bound to Ran-GTP on its own. In the cytoplasm, Ran-GTP is converted to Ran-GDP and β released for the next cycle. Most of the viral proteins make use of this mechanismn. The Hexon protein is an exception as it does not posess an NLS. In this case the NLS is complemented by pVI that binds to the hexon via hydrophobic interactions at its nuclear export signal (NES). Together they are shuffeld into the nucleus. There the complex dissasociates and the pVI is either processed by the adenoviral protease (AVP) or shutteled back to the cytoplasma via its NES and the help of the export complex. If processed, pVI looeses its NLS and the NES is disrupted. Furthermore, the binding affinity for Hexon is improved, priming the protein for its role during virion assembly. Redrawn and adapted from (Wodrich et al., 2003) with additional information from (Hodel et al., 2001; Weis, 2003).

Capsid assembly

Having all the necessary proteins at the ready, the virions are assembled. As is the case with the majority of icoshaedral viruses, adenovirus is believed to frist assemble the empty capsids and then to insert the genome. The process starts with trimerization of **Hexon** and the interaction with **Penton** proteins. **Hexon** trimerization is supported by the chaperon like protein **L4 100k**. Subsequent addition of the minor capsid proteins, **IIIa**, **VIII**, and **IX** and non structural proteins follows. One of the main

pruposes of the minor capsid proteins, also called cemet proteins, is to interact with Hexon and Penton proteins to further stabilize the virion (Ahi & Mittal, 2016).

Genome packaging and maturation

The viral genome is specifically recocnized by its packaging sequence. The packaging proteins IVa2, L4 33K, L1 52/55K, L4 22K, IIIa and cellular proteins bind to the domain and transport the DNA into the capsid. Inside, the genome is assoviated with the core proteins V, VII and μ.. Protein V builds the "shell" of the viral core and μ, being the processed form of pX (Leppard, 2008), condenses the genome together with other viral proteins (Ahi & Mittal, 2016). Finished virions cluster together and from organized, thighly packed areas within the nucleus (Georgi & Greber, 2020). Maturation of the virus relies on the adenoviral protease (AVP). Precursor proteins are cleaved and converted into their final form - the virion becomes infectious. It is now in metastable state, ready for uncoating in the next host cell (Mangel & San Martin, 2014).

Release of viral particles

The final step of the infection cycle, is the release from the host cell. Various virus-encoded proteins can cause cell death and thus lysis of the host cell (Flint & Nemerow, 2017). One gene is of particularly interest. The E3 encoded 11,6K adenvorius death protein (ADP). Although ADP is part of an early transcription unit, it is only present in very small quantities at the beginning of the infection. In the late phase, due to expression enhancement by L4 33K and L4 100K, it is highly abundant. It is believed that ADP direclty leads to permeabilization of the nuclear membrane, thus releasing the virions from the nucleus and subsequently from the host cell (Georgi & Greber, 2020; Tollefson et al., 1996).

A non-lytic pathway for egress would be via cell extracellular vesicles. They are membranous vesicles used in cell-cell comunication. Virues, including adenovirus, can highjack these vesicles and use them for tramsmission and intercellular spread (Ipinmoroti & Matthews, 2020). An advantage here, is the virion's protection from blood distributed neutralizing antibodies (Dogrammatzis et al., 2020).

Another non-lytic pathway, involving cell death tough, is autophagy. Macrophages take up apoptotic cells, usually for means of recycling and orderly dismantlement. Adenovirus may induce autophagy pathways and thus make use of macrophages, internalizing infected cells, for distribution (Jiang et al., 2008).

1.1.5 Adenovirus disease

Human adenoviruses can cause a variety of diseases. An initial infection usually occurs in early childhood, usually with mild manifestation, and leads to lifelong immunity. However, in immunocompromised patients or in a closley packed environments such as the military, adenovirus can lead to higly proplematic diseases (Lynch et al., 2011).

There is a varity of reasons why patients are immunocomprimised, such as immunodeficiencies present already at brith (congenital), transplant recipients, human deviciency virus (HIV) or patients undergoing chemotherapy. Congenital immunodeficiencies, such as serve combined immunodeficiency (SCID) syndrome are a risk factor for reccurrent respiratory infections with possible lethal outcome. The mortality is reported as high as 55% with diseminated disease, fully fledged infections, which, outgoing from one locus, affect a whole organ or even the whole body (Lion, 2014). Hematopoietic stem cell transplant (HSCT) recipients are of particular risk. Incidence of infection ranges from 3 to 47%, with higher rates in the pedriatric populations than in adults. Reported mortality rates can be as high as 53%. In immunocompromised patients after solid organ transplants (SOC), the incidence is higher and mortaly rates are up to 36%. This clearly shows the clinical significance of adenovirus (Y. J. Kim et al., 2007). Also at risk, but less so, are chemotherapy and HIV patients. Adenovirus infections in HIV patients is usually only represented by gastrointestinal related symptoms and rarely show lethal outcome. A factor most likley contibuted to effective antiretroviral treatment strategies (Huppmann & Orenstein, 2010; Lion, 2014).

Transmission

As a non-enveloped virus, the adenovirus particle is very stable to environmental influences. In a dry environment it remains infectious for weeks and is resistant to many disinfectants. For example, highly concentrated alcohol solutions with exposure times of at least 2 minutes are required to successfully inactivate the virus. This shows how important correct hygiene is, especially in the hospital setting. Transmission usually happens via inhalization of aerozols and droplets of infected persons. Furthermore, infections can also occur through direct contact of droplets with the eye, via the fecal-oral route, infected tissues or smear infection from surfaces (Kampf, 2016; Lion, 2014).

Tropismn

The infection symptoms are related to tissues affected, which are different depended on the adenovirus type (Lynch et al., 2011). Receptor tropismn alone is not sufficient to predict which organ is the target as the same receptors are found in different tissues (Greber & Flatt, 2019). More research is needed pinpointing the targets of the specific virus types and their triggered clinical pathologies **Table 1** (Ghebremedhin, 2014).

Table 1 **HAdV types, their tissue tropism and resulting diseases.** HAdV can cause an array of different diseases in different parts of the human body. In immunocompetent patients they usually have mild course and are self-limiting, leading to lifelong immunity. In immunocompromised hosts, the diseases can lead to life threatening complications. Adapted from: (Ghebremedhin, 2014)

HAdV Species	Type	Type of infection
A	12, 18, 31	gastrointestinal, respiratory, urinary
B	3, 7, 16, 21, 11, 14, 34, 35	keratoconjunctivitis (first four), gastrointestinal, respiratory, urinary
C	1, 2, 5, 6	respiratory, gastrointestinal including hepatitis, urinary
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49	keratoconjunctivitis, gastrointestinal
E	4	keratoconjunctivitis, respiratory
F	40, 41	gastrointestinal
G	52	gastrointestinal

Clinical presentations of **respiratory** infection are fever, pharyngitis, tonsilitis, cough and sour throat. Accompanying gastrointestinal symptoms are possible (Lynch et al., 2011). In children, adenovirus accounts for about 20% of respiratory infections (Esposito et al., 2016). **Keratoconjunctivitis**, the infection of the cornea and the conjunctiva, the tissue inside of the eyelids, is one of the most frequent infectious ocular diseases. Adults are most often affected but in general, all age groups are susceptible. Most common adenoviral related clinical manifestation is epidemic keratoconjunctivitis. Symptoms range between, redness of the eye, photophobia, and foreign body sensation. Subsequent pharyngoconjunctival fever follows and shows itself by pharyngitis, rhinitis, fever, and regional lymphoid hyperplasia. It is believed that less than 50% are diagnosed correctly because of bias towards antibiotic use. If needed, treatment is symptomatic only, also attributed to the fact, that there is no approved specific medication (Jhanji et al., 2015). **Gastrointestinal** (GI) involvement usually is a by-product to an adenovirus infection in a different location. However, some types have displayed preference for the GI tract. Observed symptoms are gastroenteritis or diarrhea, but also rare symptoms like haemorrhagic colitis or hepatitis are possible (Lynch et al., 2011). Adenovirus can also

affect the urinary tract, in particular in immunosuppressed renal transplant recipients. This may lead to acute graft rejection, systemic spread of infection and in the worst case, death via multiple organ failure (Nanmoku et al., 2016).

Latent infection

Besides the common course of an infection, ending in complete clearance of the virus, HAdV are capable of establishing a persistent foothold within the host. Adenovirus in latent stage is reported for various areas of the body including tonsils, adenoids, lymphocytes (especially those of the GI tract) and lung (Garnett et al., 2009; Roy et al., 2011; Zhang et al., 2010; Zheng et al., 2016). The molecular process in its complete extent still remains elusive. However, interferon (IFN) has been shown to suppress the adenoviral lytic cycle in human lung epithelial cells. IFN-alpha and IFN-gamma were able to block viral replication by increasing the interaction of the tumor suppressors RB and p107 with E2F. Subsequent binding of the arising complex to the E1A enhancer region reduced E1A expression and thus expression of all following viral genes (Zheng et al., 2016). Persistent adenoviral infection was also thought to be a factor in chronic obstructive pulmonary disease (COPD). However a clear indication for solid persistence could not be provided (McManus et al., 2007). Persistent infection is of particularly interest in immunocompromised individuals as it can serve as reservoir for the next disease outbreak. For instance, the viruses can be reactivated from lymphocytes in the GI. This leads to the GI epithelium being targeted first and from there, the entire body. The risk of that happening is highly relevant, thus it is suggested to check the viral load in the intestine prior to HSCT therapy (Kosulin et al., 2016; Lion, 2019).

1.1.6 Host defense and treatment options

Host defense

The immune system (IS) has evolved a multitude of ways to defend the organism from incoming pathogens. As viruses need host cells to replicate, they need to enter them. Therefore, the first line of defense are **mechanical barriers** of epithelia, skin and mucosal surfaces and the chemically hostile environment of the gut e.g., preventing access to human tissues. Having passed this hurdle, viruses face numerous parts of the innate immunity. **Pattern recognition receptors** detect pathogens or their products. For example, Toll-like receptor (TLR) 9 recognizes dsDNA, and triggers, like other members of its receptor family, inflammatory responses, mediated trough **interferons** and **cytokines** (Atasheva et al., 2019; Mueller & Rouse, 2008). Furthermore, the virus is recognized by natural killer cells (NK), macrophages, dendritic cells (DC) and the complement system (Greber & Flatt, 2019).

Uninfected cells are not attacked by **NK cells** as they provide negative signals such as high expression of MHC molecules together with autologous peptides on their surface. Two classes of **MHC molecules** are important here: class I and class II. Both present peptides on the cell surface. The peptides are obtained through uptake from the cell itself or the extracellular space. In general, class I presents self-peptides and class II primarily external ones. Class I is ubiquitously expressed in the body and serves as "I am healthy" message to immune cells, while class II is found on specialized leukocytes. Leucocytes, such as **macrophages** or **dendritic cells**, actively sample their adjacent area and take up antigens for subsequent presenting, thus their name, **antigen presenting cells** (APC). The external material is either taken up through engulfing (phagocytosis) or receptor mediated endocytosis. If a NK cell encounters a cell with low levels of MHC class I, common in viral infection, as viruses often inhibit the MHC presentation, it attacks and destroys the cell via excreted membrane disruptive granules. This is called missing self-recognition and a highly important function of NK cells. Other immune cells such as **CD8 cytotoxic T cells** need the MHC as clue and can't react to its absence. MHC class II on the other hand is used as means of education for other immune cells, especially from the adaptive immune system such as **CD4 T cells** and **B cells**. Thus, the innate prepares the transition to the adaptive. Antigens are taken up, processed, and transported to lymph nodes. If a foreign peptide is presented, interacting lymphocytes are activated and the adaptive immune response triggered (Mueller & Rouse, 2008; Punt et al., 2019). CD4 T cells with their functions of activation and expansion of CD8 T-cells and

B cells, as well as their memory and cytotoxic function play a central role in the response to adenovirus infection and its clearance from the body (Chatziandreou et al., 2007; Doherty et al., 2018).

The complement is a set of proteins that interact with the innate and the adaptive immune system. When activated it can form membrane disruptive channels, thus lysing microbial pathogens directly or opsonise them for destruction (Rambach et al., 2008). The uptake of complement marked virions protects the cell, as endosomal escape is prevented (Greber & Flatt, 2019). Adenovirus opsonised by immunoglobulin M (IgM) and the complement is taken up by Kupffer cells (macrophages of the liver) and thus rapidly cleared from the blood stream. IgM is a polyreactive antibody of the innate immune system and a very effective activator of the complement pathway. While its monomeric binding affinity is lower than that of highly specific IgG antibodies of the adaptive IS, they are available right from the start of an infection. In multimeric complexes, binding affinity is increased which is especially useful for repetitive structures such as viral capsids (Z. Xu et al., 2008). Besides cellular mediated immunity, highly specific antibodies IgG are of central importance for the organism's protection, particular those that are classified as neutralizing antibodies. Only a small subset of all produced antibodies has neutralizing effect. They bind the virion in a manner that prevents infection, e.g., by blocking parts necessary for entering a host cell. As no virus progeny can be produced, the virus cannot be disseminated in the community. Concerning RNA interference, highly important in plants and arthropods e.g., it only plays a minor role in viral defense in vertebrates (Payne, 2017).

Treatment options

If the immune system is insufficient to defeat an adenovirus infection, the question arises if there are medical treatments available. If yes, the next question is when to start the therapy. Prophylactic approaches have advantages and disadvantages. On the one hand, available antiviral therapies show limited efficacy and toxicity, on the other hand, patients may die from disseminated diseases within a few days. One option is to measure the viral load in stool samples and to start treatment above a certain threshold. As deliberately induced immunosuppression is a major risk factor, reduction of the same should be the first line action whenever possible (Lion, 2019).

Virostatic agents

The therapy with virustatics is mainly based on the nucleoside analogue **cidofovir** (CDV). Other drugs, often discussed in this context, such as ribavirin and ganciclovir, unfortunately showed no significant effect in studies (Dodge et al., 2021). The viral DNA polymerase integrates the active metabolite of cidofovir, cidofovir diphosphate, into the replicating DNA chain instead of cytidine. This leads to inefficient further elongation or, at higher concentrations, chain termination. Furthermore, incorporated CDV is not easily removed by the polymerase's exonuclease activity and, likewise at high concentrations, CDV is able to inhibit the polymerase directly. However, acquired mutations in the adenoviral polymerase can provide resistance to the drug (Chamberlain et al., 2019). All this happens at concentrations well below those necessary to inhibit cellular polymerases, leading to a desired bias for viral polymerases (Hitchcock et al., 1996). Disadvantages include low bioavailability, over 90% are excreted unchanged via the urine, and significant nephrotoxicity. Cidofovir is primarily cleared via the kidneys, which are quick in uptake but slow in release, leading to high drug concentrations. Damages can go as far as lifelong dialysis becoming necessary (Caruso Brown et al., 2015; Dodge et al., 2021; Hibbert et al., 2018; Vora et al., 2017). Furthermore, the reconstitution of the immune system still is required for complete virus clearance **Figure 10** (Hiwarkar et al., 2017).

A newly described, very promising and already used medication is **brincidofovir** (BCV). It is a lipid conjugated prodrug of cidofovir. It has improved oral bioavailability and higher intracellular concentrations of the active metabolite. In contrast to CDV, BCV is not a substrate for the kidney localized organic anion transporter 1, thus nephrotoxicity levels are greatly reduced or even absent. Main dose limiting side effects are gastrointestinal, including decreased appetite, cramps and diarrhea. The symptoms usually resolve after end of treatment. This proofs BCV as great new candidate for treating adeno viremia **Figure 10** (Alvarez-Cardona et al., 2020; Hiwarkar et al., 2017; Londeree et al., 2020).

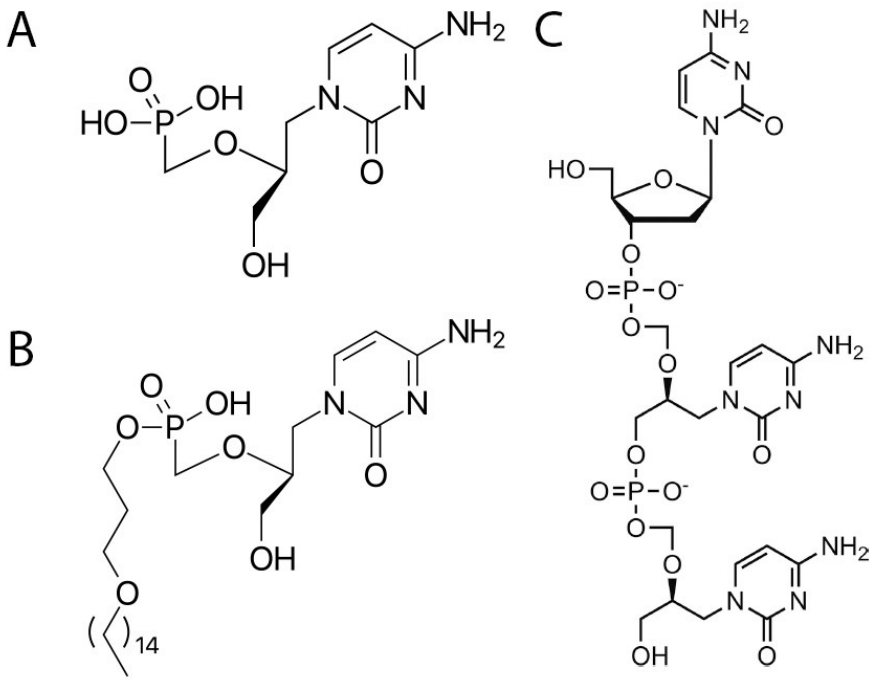


Figure 10 The virostatics cidofovir and brincidofovir. A: Chemical structure of the cytidine analogue cidofovir. It is the treatment of choice if reduction of an induced immunosuppression is not possible. Treated patients experience a significant reduction in adenoviral load and improvement of adeno viremia. Unfortunately, cidofovir displays low bioavailability and high nephrotoxicity. B: Brincidofovir is a lipid conjugated prodrug of cidofovir. It shows higher bioavailability, higher intracellular concentrations, and lower or absent nephrotoxicity. The active metabolite is the same as for cidofovir: cidofovir diphosphate. C: Two cidofovir molecules incorporated 3' of a cytidine nucleoside. The incorporation either leads to inefficient elongation or, at higher concentrations, chain termination. Furthermore, at higher concentrations, cidofovir can inhibit the viral polymerase directly. Adapted from: (Chamberlain et al., 2019; Wikipedia, retrieved 2022) with additional information from: (Alvarez-Cardona et al., 2020; Hiwarkar et al., 2017)

Immune therapeutics

The clearance of adenovirus from the body is predominantly facilitated by T-cells, CD4 T cells for that matter. Application of these cells in treatment approaches was successful in HSCT recipients. Here, primary T-cells are isolated from the transplant donor's blood and stimulated with adenovirus antigens *in vitro*. The thus activated T-cells are isolated by rapid immunomagnetic selection of IFN-gamma-secreting cells and given to the transplant recipient. In order to not damage the graft, source of the T-cells needs to be the transplant donor, or if not available, a haploidentical third party. Graft versus host disease of course is a possible risk but does not seem to be of contraindicating probability (Chatziandreou et al., 2007; Qian et al., 2017).

Vaccines

As noted before, serious respiratory disease can also manifest in immunocompetent hosts when in close quarters. The United States military did struggle with high rates of adenovirus mediated hospitalisations, especially in trainees. To prevent future outbreaks, the army developed viral vaccines against HAdV type 7 and 4. The live, oral, enteric-coated vaccines were highly successful in providing stable immunity (Top, 1975). Unfortunately, production by the sole manufacturer stopped 1996 and stores were depleted by 1999. In the years following, the incidence rose again. After a 12 year gap the vaccination program was resumed end of 2011 (Potter et al., 2012; K. L. Russell et al., 2006). A similar experience is reported by the Korean Military were HAdV type 55 lead to several outbreaks. A vaccination program is proposed there also (Ko et al., 2021).

Arising methods

Even though playing a subordinate role in the endogenous antiviral host defense, reprogrammed **RNAi** can be employed against adenoviral replication. It has been shown that siRNA as well as miRNA mediated RNAi, targeting key adenovirus genes, can successfully inhibit adenoviral replication *in vitro* (Ibrisimovic et al., 2013; Kneidinger et al., 2012). Furthermore, the **genomic engineering tool** CRISPR/Cas9 and its family is coming more and more into focus as an antiviral therapy, making use of its inherited biological function – an adaptive defense mechanism against invading genetic elements (C. Lee, 2019; Zhang, 2020).

Last but not least, a **combination of the methods** mentioned above may also be promising. Combination therapy, already the norm for HIV, may have multiple advantages. The concentrations of the individual components can be lower, thus reducing undesirable side effects. In addition, the risk of resistances is lower and drugs, that alone are not effective enough, may be so in combination. Synergetic effects in which the observed result goes beyond the pure additive effect would be particularly desirable (Dodge et al., 2021).

1.1.7 Adenovirus as delivery vector

Adenovirus is not to be seen as a solely negative pathogen. On the one hand, there is a lot to be learned from its biology, as elaborated in chapter **1.1.2**, and on the other, adenovirus possesses various characteristics that can be exploited. The viral protein **E4Orf4** induces cancer-selective cell death outside the context of adenoviral infection. Disruption of cellular pathways that are essential for cancer cells is the likely reason. (Kleinberger, 2020). **E4Orf1** reduces the need for endogenous insulin and may be an interesting addition to diabetes therapy. In an infection, it promotes cellular glucose uptake in the absence of insulin, which would be a desirable ability in the context of insulin resistance and blood glucose level control (Peddibhotla et al., 2019).

History of gene therapy

In the first place of adenovirus properties that scientists make use of, however, is the efficient transport of genetic information. The idea of gene therapy goes back to the earliest times when the genetic basis for diseases and their inheritance became understood. The vision was to be able to cure all diseases as long as the molecular basis is known. In 1970 the first human trial was carried out. Intravenously applied Shope papillomavirus should have reduced blood arginine levels in hyperargininemic patients, through its arginase. Unfortunately, no reduction in levels could be observed. In 1980 the idea of retrovirally mediated gene therapies followed. The stable integration into the host genome was seen as great advantage. Even so, the principle was not yet completely understood (Escors & Breckpot, 2010). One of the first and best-known successes, albeit controversial, was in the year 2000. Severe combined immunodeficiency-X1 disease was treated by *ex vivo* transduction of patients CD34 cells with gamma c cytokine receptor encoding retrovirus and subsequent reinfusion. Underlying cause of the disease is a genetic defect in this very receptor, causing lymphocytes to be blocked early in their development. The disease was successfully treated but patients developed leukaemia as the virus integrated near proto-oncogenes, genes involved in cell proliferation (Bushman, 2020; Cavazzana-Calvo et al., 2000). In the following years the field moved on to the less oncogenic retroviruses like lentivirus and other viral vectors, such as recombinant adenovirus (rAd), adeno-associated virus (AAV) and herpesvirus for gene transfer (Escors & Breckpot, 2010; Gruntman & Flotte, 2018). Furthermore, non-viral delivery methods of genetic material, e.g., lipid-based transfer, are also becoming increasingly important, exemplary seen at the novel SARS-CoV-2 mRNA-based vaccines (Buck et al., 2019; Polack et al., 2020).

Adenovirus mediated gene transfer

Advantages, disadvantages, and safety

Adenovirus has many promising characteristics in the context of genetic transport. It shows extremely efficiency transduction of a large number of different cell types, both replicating and quiescent, together with high transgene expression. Furthermore, their deployment is well established by now, with high experience in clinical administration, safest dosing and application routes (C. S. Lee et al., 2017). Adenovirus vectors are the most used in current clinical trials (Wiley, retrieved 2021). There is a vast array of different species and types with different tissue tropism. Recombinant adenoviruses are replication deficient and their safety profile highly satisfying as well (C. S. Lee et al., 2017). As adenovirus transduces its genome in an episomal manner, oncogenic concerns are greatly reduced. However, genomic integration is not unheard of. In relation to spontaneous mutation rates the risk of integration through recombination seems comparable low but indeed not impossible and therefore needs to be kept in mind (Stephen et al., 2010). Downside of non-integrated transgenes is their reduced durability, especially in dividing cells (C. S. Lee et al., 2017).

Another factor is the immunogenicity of the vector. Gene therapy can often be referred to as one-time treatment not only because one dose is enough, but also because of the subsequent, if not present already before, established immune defense against the delivery vector (Goswami et al., 2019; Samelson-Jones et al., 2020). Due to adenovirus high abundance in the community, pre-existing immunity in patients is possible. If a patient already has an acquired immune response against the delivery-adenovirus, it naturally affects the therapy's efficiency (C. S. Lee et al., 2017). Additional, residual gene expression of viral genes my lead to the elimination of successfully transduced cells (Alba et al., 2005). There are ways however, to respond without having to exclude these patients. The immune system can be suppressed temporarily, or blood-antibodies filtered out by plasmapheresis. Still, both options have downsides - being either time consuming or side effect prone - and patients with high antibody titers remain a problem. More strategies are currently investigated such as (i) transgene optimization (lower doses needed,…) or (ii) generation of less immunogenic delivery vectors. Further possibilities are (iii) to hide the viral particle by cloaking it with exosomes or silica nanoparticles, or (iiii) to alter the viral capsid to counter pre-existing immunity. Unfortunately, even using less immunogenic vectors, the capsid, expression from residual viral genes and cells presenting viral antigens may still trigger an immune response. (Dogrammatzis et al., 2020; Gao et al., 2019; Sapre et al., 2019; Sumida et al., 2005; The-Scientist, retrieved 2019). On the positive side, prevention

of immune interference is critical only for a specific limited time point surrounding the vector administration (Samelson-Jones et al., 2020).

Immunogenicity can also be beneficial. Oncolytic adenoviruses, genetically altered to be specific for tumour cells by changing promoters e.g., can lyse infected target cells and elicit an efficient immune response (Choi et al., 2012; Goswami et al., 2019). In the development of vaccinations, too, the high immunogenicity of adenoviruses, besides efficient delivery of the genetic code of the pathogen of interest, is important, as it triggers a large number of immune reactions. It must be noted decidedly however, that in case of vaccinations it is a fragile equilibrium. On the one hand triggering the immune system is intended, on the other, a too strong or even excessive induction can lead to a catastrophic outcome for the patient. In the context of HIV vaccinations, adenovirus seems to enhance rather than protect from infections. In addition, adenovirus-based vectors are able to set off severe immunopathology's such as cytokine storm syndrome, disseminated vascular coagulation, thrombocytopenia, and hepatotoxicity. These outcomes have been reported in the context of high dose intravascular application of gene therapies. In vaccinations with usually low dose intramuscular injections, the risk is significantly lower of course. However, in worldwide mass vaccinations, with an enormous number of people taking part and the difficulty of keeping person specific subsequent medical observation, it is ethically and clinically indicated to keep a close eye also on rare issues (Atasheva et al., 2019; Chang, 2021; Destefano et al., 2018; WHO, retrieved 2022). Furthermore, as is the case in gene therapy, immune response, pre-existing or acquired after the first dose in a homologous prime boost regime, against the delivery vector can reduce the efficacy of the vaccination (Chang, 2021). Novel SARS-CoV-2 vaccines are evading this problem by using rare adenovirus vectors for immunization. ChAdOx1 nCoV-19 is using a chimpanzee derived virus in a homologous two dose regime, Ad26.COV2.S a rare human adenovirus type with one dose applied and Gam-COVID-Vac, rAd26 in the first- and rAd5 in the second dose (Abbink et al., 2007; Logunov et al., 2021; Ramasamy et al., 2020; Stephenson et al., 2021). Vaccination with ChAdOx1 nCoV-19 uses a two-dose regime with the same vector in contrast to Gam-COVID-Vac. High vector specific antibody titers are reported before the second dose, thus indicating a possibly reduced efficiency of the subsequent homologous booster dose as noted above (Logunov et al., 2021; Ramasamy et al., 2020).

The three generations of recombinant adenovirus vectors

There are three generations of adenovirus vectors Figure 11. None of them are replication competent as essential genes have been removed. The vacated area can be used for insertion of transgenes. In the following generations, more and more genes were deleted, reducing immunogenicity and increasing the safety and transgene capacity of the vector. The first generation is missing genes *E1* and *E3*, in the second, deletion of *E2* and *E4* followed. (Alba et al., 2005; C. S. Lee et al., 2017). Expression of the remaining adenoviral genes, in first- and second-generation vectors, still triggers an immune response. Amplified in packaging cell lines such as HEK293, complementing missing genes, recombination between homologues sequences in the vector and the cell line are a possibility, leading to undesired replication competent virions (Alba et al., 2005). The third generation are the gutless or helper-depended viruses. Being devoid of all viral genes, with only the ITRs and the packaging signal remaining, they have a greatly increased capacity and greatly reduced immunogenicity. The possibility of an immune response against the capsid itself remains, tough (Gao et al., 2019). Capacity goes up to 36kb. Transgene expression cassettes, usually designed to be small in size, need to be elongated with non-coding "stuffer" DNA to fill up the space, as only genomes of 75-105% the wild-type size are packaged efficiently. Coding regions, repetitive sequences, toxic or immunogenic regions or hot spots for recombination should be, amongst others, avoided as stuffer DNA. The gutless' amplification helper virus is usually a first generation rAd in addition to cells expressing *E1*. Helper virus- and replication competent adenovirus contamination in the final preparation is the main obstacle, however. Various tactics are applied to reduce such a contamination. Widley applied is a CRE loxP depended method that leads to excision of helper virus' packaging signal in cells expressing CRE. Only the gutless genome, retaining the signal, is packaged into the virus progeny. In addition, inversion of the packaging signal decreases recombination events. Still the system remains imperfect (Alba et al., 2005). New helper plasmid-based approaches are reported showing no contamination. Besides being DNA only, the helper plasmid is devoid of ITR and packaging signal to prevent recombination (D. Lee et al., 2019).

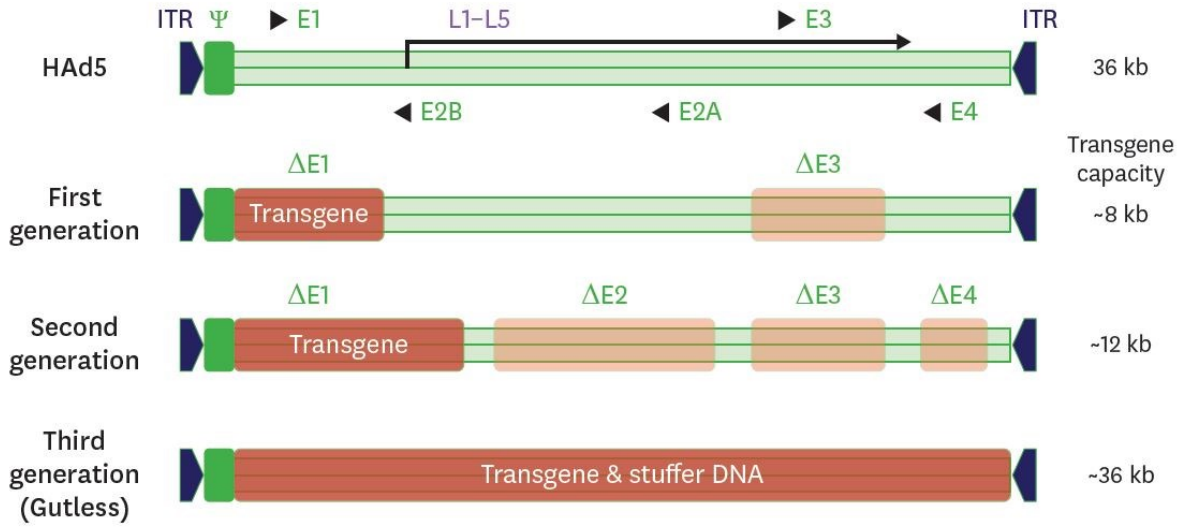


Figure 11 **Three generations of recombinant adenovirus vectors**. Essential genes for replication are missing in all generations, rendering them replication incompetent. In the first generation *E1* and *E3* are removed, followed by *E2* and *E4* in the second. The third generation, also called high capacity or helper depended adenovirus is devoid of all genetic elements except the packaging signal and ITRs. The place freed up by deletions is used for transgene insertion. Whereas the capacity of the first generations is limited, however, compared to other vectors, still comparable high, the capacity of the helper depended adenovirus is up to 36kb. Therefore, transgene expression cassettes, usually too big, become too small. As the capsid packaging is efficient only for genome sizes of 75-105% of the wild-type, stuffer DNA needs to be introduced to fill up the space. First- and second-generation vectors can be amplified in packaging cells supplementing the missing genes. The third generation needs a helper virus in addition. Advantages of the helper dependent virus is the high capacity and greatly reduced immunogenicity. Contamination with recombination competent vectors (issue in all generations) or helper virus in the final preparation still remains an obstacle. Figure from: (Chang, 2021) with additional information from: (Alba et al., 2005; Gao et al., 2019)

1.2 Genomic engineering tools and their principle

Genetic engineering tools, having reached their current peak in the CRISPR/Cas9 system, have become an integral part of molecular biology. The ability to change DNA at desired positions has revolutionized the field. Generating knockouts in specific genes to study their function or treatment of genetic disorders are prominent examples (Knott & Doudna, 2018).

1.2.1 Zinc finger nucleases

So-called programable nucleases existed before CRISPR/Cas was discovered. Zinc finger nucleases (ZFNs) are the first to be mentioned here. Different zinc finger motifs are put together, each of which recognizes a specific DNA base triplet. At the C-term is the nuclease FokI which cuts the DNA strand. For cutting, the nuclease needs to dimerize, which lengthens the recognition site and therefore specificity. However, also with only one complex bound to DNA, FokI may dimerize and intruduce off-target effects. Furthermore, not all possible base-triplets can be targeted and the production of highly specific and cutting-efficient complexes with low cytotoxicity is difficult **Figure 12** (H. Kim & Kim, 2014).

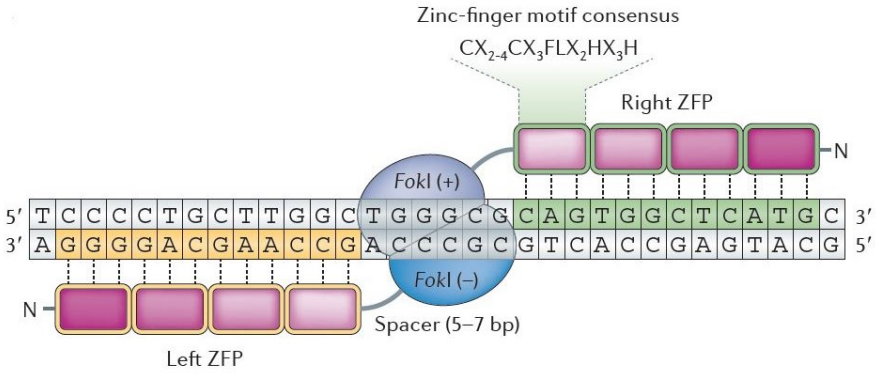


Figure 12 **Zinc finger nucleases (ZNFs)**. ZNFs consist of a DNA binding Domain and a nuclease domain. The zinc finger motifs are responsible for sequence specific binding. Each motif recognizes one DNA nucleotide triplet. The bound nuclease, thus directed to the desired location, cuts after dimerization with another FokI domain. Required dimerization increases the specificity as it increases the recognition length. However, FokI may also dimerize with only one ZNF complex bound to the DNA, leading to off-target effects. Producing efficient complexes with low cytotoxicity remains a challenge. Adapted from: (H. Kim & Kim, 2014)

1.2.2 Transcription activator-like effector nucleases

The next step in evolution are the transcription activator-like effector nucleases or short TALENs. Similar to ZNFs, a DNA sequence recognition domain is fused C-term to the nuclease domain FokI. However, the recognition domain consists of 33-35 amino acid long repeats - the transcription activator like effectors (TALEs). Each repeat is responsible for the binding of one DNA nucleotide. Which one it is, is determined by the two amino acids at positions 12 and 13, repeat variable residues (RVDs). TALENs can be constructed to target almost any desired sequence, a big advantage over the other engineering tools. Still, the construction of the arrays is challenging and time consuming and, due to the highly homologous sequences of the amino acid repeats, recombination in transduced cells likely **Figure 13** (H. Kim & Kim, 2014; Xiong et al., 2015).

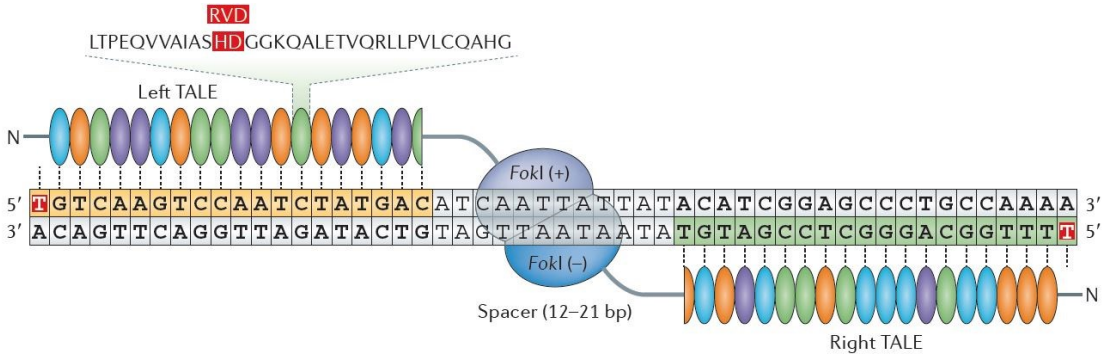


Figure 13 **Transcription activator-like effector nucleases (TALENs)**. As is the case in ZNFs, TALENs consist of a DNA recognition domain and the nuclease domain FokI, that cuts the DNA at the destined position. The recognition domain consists of 33-35 amino acid long repeats with variable residues at position 12 and 13. These residues determine which DNA nucleotide is bound. Almost any desired DNA sequence can be targeted. However, construction of the arrays is demanding, and the highly homologous repeats can lead to recombination in transduced cells. Adapted from: (H. Kim & Kim, 2014)

1.2.3 The CRISPR/Cas system

History

Clustered regularly interspaced short palindromic repeats (CRISPR) were first mentioned in 1987. Researchers discovered unusual repetitive sequences in the genome of *Escherichia coli*. Their subsequent discovery in various other bacteria and archaea, highlighting their conservation and therefore a putative evolutionary import function. Uncovering of the link between the CRISPR associated (Cas) proteins and the CRISPR domain followed. In 2000, sequences split by CRISPRs, were shown to be derived from bacteriophages, archaeal viruses and plasmids which gave the final clue to the domain's function (Ishino et al., 2018). It is an adaptive immune system, protecting the organism from exogenous genetic elements. The Cas proteins are directed by short RNAs in a sequence specific manner to cut and thus silence invading genetic elements (W. Y. Hwang et al., 2013). Exploited in molecular biology research this has enormous potential. The breakthrough came in 2012 with the description and optimization of the CRISPR/Cas system as a genomic engineering tool. A Cas nuclease can be directed to DNA locations of interest simply by providing short RNAs as guide. (Jinek et al., 2012). Compared to the bothersome production of ZNFs and TALENs this has significant advantages (Xiong et al., 2015). The magnitude of this technology for science was confirmed by being awarded the Nobel Prize in chemistry in the year 2020 (TheRoyalSwedishAcademyofSciences, retrieved 2020)

Types

Cas systems are found in about 45% of bacteria and 85% of archaea. Since their first discovery many additional *cas* loci have been described. They are classified by their effector gene into class I and class II and subsequently into six types (I-VI) and further subtypes (McGinn & Marraffini, 2019). No simple classification is possible as there is no single shared protein amongst the types. Instead, several characteristics are used: signature *cas* genes that are specific for individual types, phylogeny of the most highly conserved protein across species (Cas1) and the gene organisation of the loci. Class I consists of the common and diversified types I, III and the rarer type IV. Effector modules, responsible for binding RNA guide and target, of class I contain an extravagant set of different Cas proteins compared to the simpler organisation of class II systems. Class II consists of type II, including the scientifically widely used Cas9 effector protein, V and VI. Their effector modules consist of a single large multidomain and multifunctional protein Figure 14. Most types target and cleave DNA, but there are also types that target RNA in addition to DNA (Samai et al., 2015; Strutt et al., 2018; Zhu et al., 2018), whereas exclusive targeting of RNA is primarily found in class II type VI (Makarova et al., 2018).

The CRISPR/Cas locus and the two classes

The CRISPR/Cas locus consists of ***cas* genes** and the **crispr array**. The *cas* genes, either a diverse set as in class I or primarily one single multifunctional gene as in class II, are responsible for implementation of the genetic defense response. The crispr array, usually found upstream of the *cas* genes, forms the immune memory from which the guide RNAs are transcribed. Inside are spacers, short fragments of invading genetic elements, split by clustered regularly interspaced short palindromic repeats (CRISPR). The array is marked by a 5' leader sequence that regulates the array's transcription and is used during the spacer integration process **Figure 14** (Garrett et al., 2020; Makarova et al., 2020).

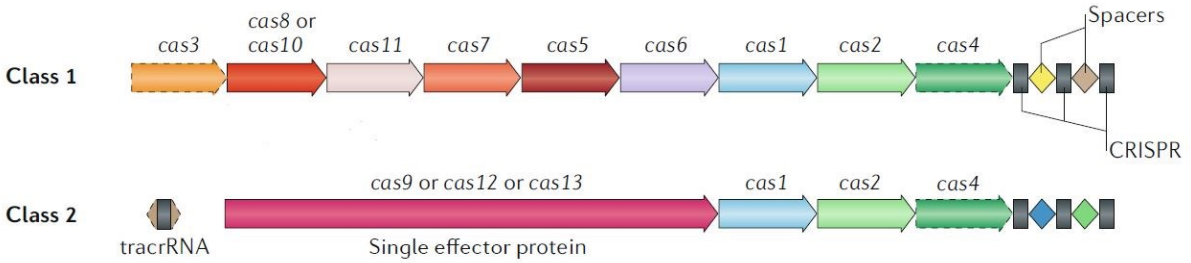


Figure 14 **CRISPR/Cas locus and classes of cas systems**. Cas systems are split into two classes, six types and subtypes. Classification is no easy task as there is no single protein shared between all types. Therefore, it relies on other characteristics such as genomic organisation of the *cas* loci. The effector module of class I consists of various proteins marked in shades of **red**, whereas in class II one single multifunctional effector protein is encoded (**pink**). Cas modules in **light blue and green** are involved in spacer integration (Dashed lines indicate dispensable or missing parts in some subtypes). Target silencing is mediated by **orange** marked cas3 or executed by multifunctional proteins. The transactivating CRISPR RNA (**tracrRNA**) found in class 2 takes part in the interference process by forming a complex with the effector proteins. Usually found downstream is the CRISPR array. It is the immune memory through integrated spacers, short fragments of invading genetic elements, split by CRISPR. Adapted from: (Makarova et al., 2020)

Principle

It has advantages for bacteria and archaea to be susceptible to external genetic material. It grants them access to evolutionarily advantageous properties such as antibiotic resistances for instance. At the same time, however, the organisms become more susceptible to harmful parasitic elements. This is why bacteria and archaea have evolved several measures to counter this disadvantage. The CRISPR/Cas system is one of them (McGinn & Marraffini, 2019; Thomas & Nielsen, 2005).

Adaption and spacer acquisition

First step in the immune response is **adaption** by incorporating **spacers** of foreign genetic elements into the **crispr array**, thus forming the immune memory. The process makes use of the DNA repair machinery and Cas1 and Cas2 which are highly conserved amongst most CRISPR/Cas types. Free dsDNA ends are bound and spacer acquisition from ds breaks is stimulated. Uptake of autologous spacers from the host chromosome, needs to be avoided to **prevent self-targeting**. First, activity of the DNA repair machinery is slowed by eight-nucleotide sequence motifs, the chi sites enriched on the host chromosome. Second, at infection a phage genome presents dsDNA ends, whereas the host genome is circular. Third, spacer acquisition is a very rare event seemingly balancing the benefits with the danger of autoimmunity (McGinn & Marraffini, 2019). Adaption can be **naïve or primed**. In case of naïve adaption, no available spacer matches the incoming invader, therefore new spacers need to be obtained first. In primed adaption, when spacers match the invader (mismatches are tolerated to a certain degree) the subsequent interference response leads to cutting of the target, which provides additional dsDNA breaks for further spacer acquisition (Garrett et al., 2020).

If the fragment (**protospacer**) was successfully obtained, Cas1 and Cas2 form a complex and act as integrase. In addition, the insertion process simultaneously duplicates the CRISPRs. The complex has two DNA binding domains, one that binds the protospacer and one that binds the leader sequence of the crispr array. New spacers are primarily added to the leader-end of the array; therefore, the chronical order of invaders can be identified by their spacer position. Leader-end spacers show higher expression than the ones further downstream. The response is thus biased for recent invaders **Figure 15** (McGinn & Marraffini, 2019).

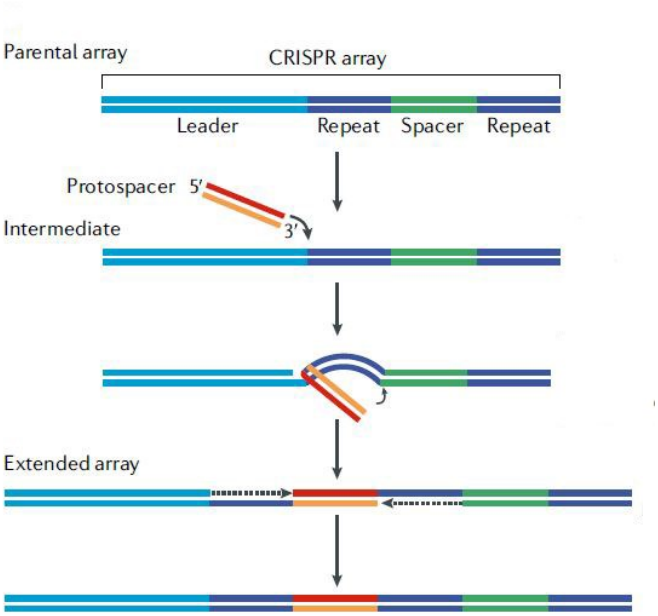


Figure 15 **Integration of new spacers into the CRIPSR Array**. Short fragments (proto spacer) of invading genetic elements are obtained with the help of the DNA repair mechanism. Cas1 and Cas2 form a complex and facilitate integration of the spacer into the array. The complex has two binding domains. One binds to the leader sequence of the array, the other to the protospacer. A nucleophilic attack by the 3'-OH of the protospacer at the leader end of the repeat follows. The same happens at the end of the previously integrated spacer. The new spacer is ligated into the array and the single strand stretches replicated, thus forming a new repeat. Adapted from: (McGinn & Marraffini, 2019)

Spacer adaptation in RNA targeting types may rely on the same genes if present or complemented from other CRISPR loci on the genome. Furthermore, spacer-processing directly from RNA is a possibility but requires a reverse transcriptase (RT) for insertion into the array. Indeed, RT enzymes where found in connection with CRISPR loci in some types (O'Connell, 2019; Toro et al., 2019; Zhu et al., 2018).

The protospacer adjacent motif (PAM)

Self-targeting may not only arise through uptake of autologous spacers but also through sequence specific targeting of the stored spacer within the CRISPR locus itself. PAMs are used as means of licensing for interference. These are short motifs flanking the target sequence in the invading genetic element. Cas4 facilitates a preference for PAM connected spacers during acquisition. As PAMs are missing in the CRISPR array no recognition or cleavage occurs (McGinn & Marraffini, 2019). However, there are types being very flexible in their PAM composition. It has been reported that in these cases, although a variety of PAMs are possible, the ones that show high similarity to the CRISPRs inhibit interference, thus severing as something like an anti-tag. The RNA cleaving Cas13 seems to work

without such a motif, although protospacer flanking site (PFS) preferences have been reported in some cases. As RNA is targeted, direct attack of the CRIPSR domain is not possible. Nevertheless, there are instances, although unlikely, where targeting of the crispr array transcript could be in antisense allowing targeting. Lateral gene transfer of the arrays is one option. It is proposed that in this case the same anti-tag mechanism may play a role (Meeske & Marraffini, 2018).

Precursor CRISPR RNA expression, processing, and complex loading

The guide RNAs that direct the effector complex to the intended target are called CRISPR RNAs (crRNAs). They are transcribed from the CRISPR array as precursor crRNAs (pre-crRNAs) in one long RNA. This is initiated from a single promoter upstream of the leader or, in some cases, individual promoters within the array. The pre-crRNA is recognized by hairpins the CRISPR form, or by sequence specific repeat binding of the trans activating crispr RNA (tracrRNA) that complements the hairpin in types with unstructured repeats. Effector proteins such as Cas6 in class I or multifunctional proteins alone, as is the case in RNA targeting type VI Cas13, or with the help of RNaseIII in case of DNA targeting type II Cas9, bind and cut the transcript into crRNAs. Each contains one target specific spacer. Subsequently, Cas effector proteins, that mediate target binding and cleaving, assemble. In case of multifunctional proteins, the complex already assembles during pre-crRNA processing and simply remains bound Figure 16 (Charpentier et al., 2015).

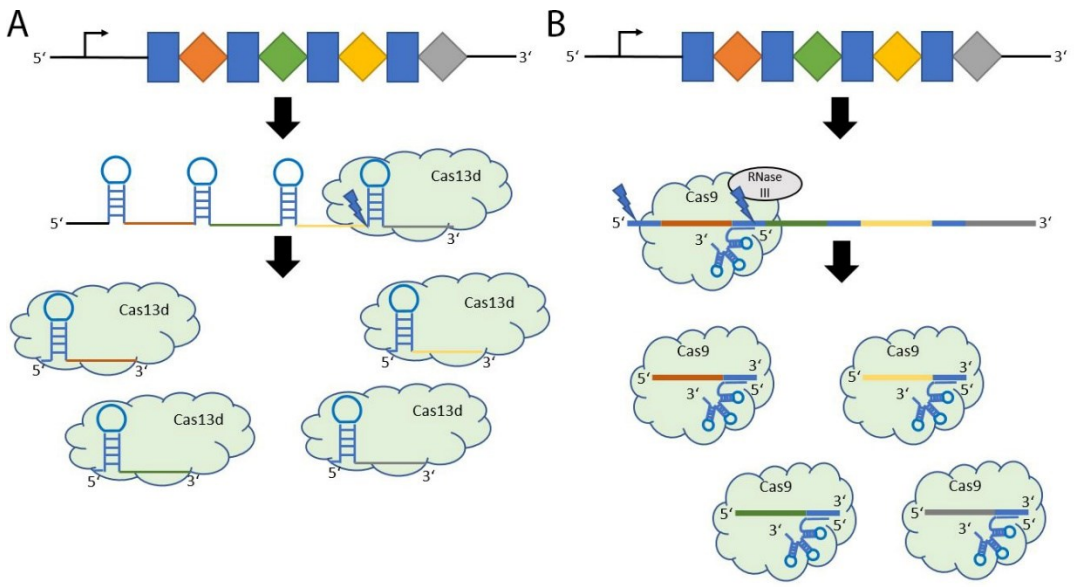


Figure 16 **Pre-crRNA processing by Cas13d and Cas9**. The pre-crRNA is transcribed as one long RNA from a single promoter upstream of the crispr array. In some cases, transcription from multiple promoters within the array is possible. Recognized via hairpin structures **(A)** of the repeats (blue) or by sequence specific repeat binding of tracrRNA **(B)** and subsequent interaction with the effector complex, the crRNA is processed, forming mature crRNA for target guiding. Processing by Cas13d involves cleaving-off part of the repeat and part of the spacer. In the case of Cas9, the first cut happens 3' by RNase III, the second removes the overhang 5'. Final result are effector complexes and crRNA with one target spacer each. Adapted and redrawn from: (Charpentier et al., 2015; Konermann et al., 2018)

Interference

The armed complex consists of different proteins, or domains in multifunctional proteins, that mediate target binding and cleavage in a sequence specific manner. Directed by the crRNA, with or without the help of a tracrRNA the spacer base pairs to the target. DNA targeting Cas9 has two nuclease domains. HNH cleaves the crRNA bound strand and RuvC the opposite. This leads to a double-strand break, exactly three base pairs upstream of PAM composed of NGG, and thus disruption of the target (Nishimasu et al., 2014). RNA targeting Cas13 has two HEPN domains that facilitate RNase activity. As soon as target RNA is bound, it is cleaved by the HEPN domains and degraded. The exact position of the cut seems not to follow a strict principle and can happen all across the targeted RNA molecule (Huynh et al., 2020). In Cas13d, bystander cleavage was reported as additional property. RNA molecules that share homologies to the target RNA can be cut collaterally. However, as this process was inefficient compared to its on-target activity it may not play a major role within the cell **Figure 17** (Konermann et al., 2018).

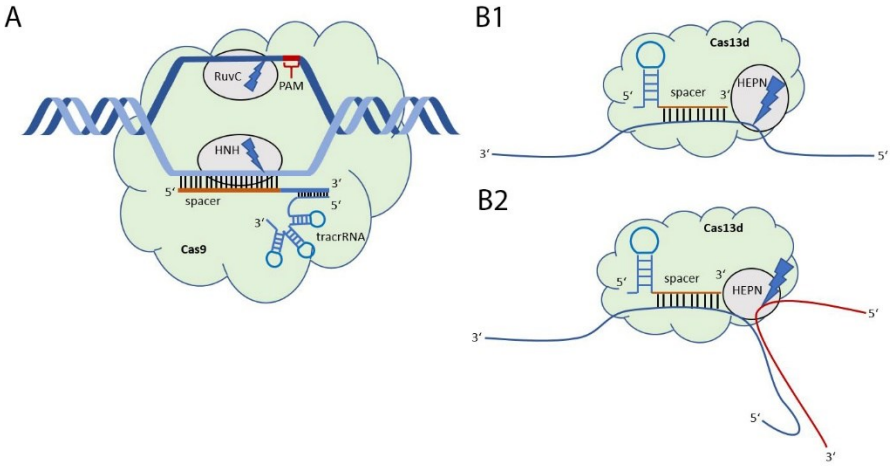


Figure 17 **Interference process of Cas9 and Cas13d**. **A**: The crRNA is bound to Cas9 via the connection of the tracrRNA. As soon as the target and PAM sequence is recognized, the spacer region of the crRNA base pairs. The nuclease domains of Cas9, HNH for paired strand and RuvC for the opposite strand, subsequently cleave the DNA. **B1**: Cas13d cleaves RNA via its HEPN domains as soon as its crRNA encounters the target sequence. **B2**: Bystander cleavage of RNAs which show homologies of the target strand has been reported but seems to not play a major role in the cell. Adapted and redrawn from: (Konermann et al., 2018; D. Wang et al., 2018)

1.3 The CRISPR/Cas system in science

The following chapter is going to concentrate on Cas9 and Cas13d (CasRx), used in this project.

1.3.1 Cas9: Adaption, properties, and safety

Adaption in Science

The class II type II protein Cas9 was the first to be adapted for genome editing. It was shown that the multifunctional Cas9 from *Streptococcus pyogenes* can be used to induce site specific double-strand DNA breaks. Furthermore, parts of crRNA and tracrRNA can be fused forming one single guide RNA (sgRNA), containing a 20-nucleotide spacer, thus increasing the method's convenience (W. Y. Hwang et al., 2013; Jinek et al., 2012).

Cas9 induced double-strand breaks are religated by the DNA repair machinery, either by end-joining or homologous directed repair. In non-homologous end joining (NHEJ) the DNA strands are simply fused together, leading to short random insertions or deletions (indels). Alternatively, fusing may also rely on homologies. These can be in close vicinity on the DNA strand itself, leading to small indels in case of micro homology end joining (MMEJ) or large deletions in single strand annealing (SSA). If a repair template is present, the break can be efficiently resolved to its original state by homology directed repair (HDR) **Figure 18** (Seol et al., 2018). All this can be exploited for genome editing. The random indel mutations of NHEJ may lead to frameshifts in a targeted protein's coding region, altering the reading frame, or even the introduction of a premature stop codon. Both likely results in a knockout, which helps in understanding a gene's function. The precise outcome of repair is difficult to predict, however. The HDR pathway is of particular interest as it gives the possibility of trans-gene insertion by providing a repair template. Insertion of reporter genes or antibiotic resistances are attractive options for example. The template herby needs to contain the transgene or any other genetic element of interest, flanked by homology arms, matching flanking sequences on the target DNA (Ata et al., 2018; A. Ran et al., 2013).

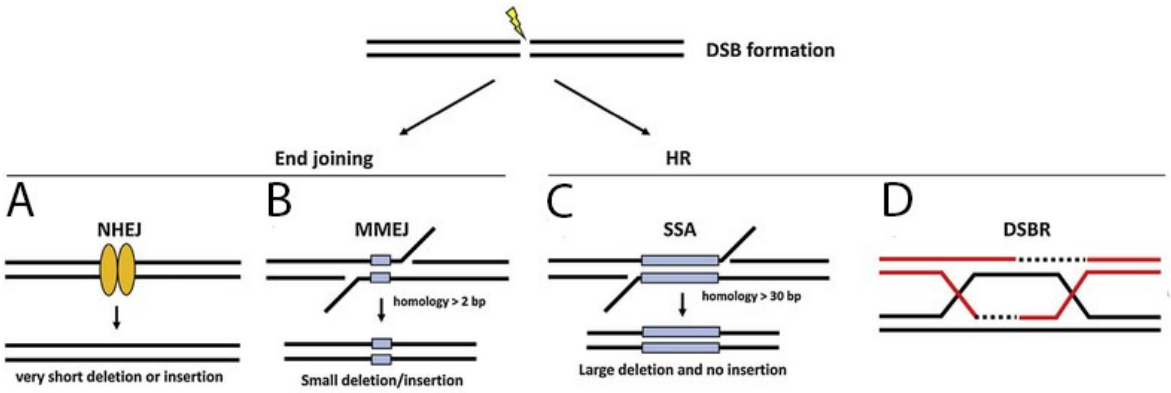


Figure 18 Pathways of DNA double-strand break (DSB) repair. The DSB can be processed in 4 primary ways. Error prone end joining, or homology directed repair. A: In non-homologous end joining the ends of DNA strands are fused, leading to short random indel mutations. This may lead to a frameshift or premature stop codon in a targeted gene's coding region, thus likely knocking out its function. B: End joining may also rely on microhomologies or large homologies (C) in vicinity of the break. This can result in big deletions. D: Homology directed double-strand break repair is the most precise method. It makes use of repair templates that share the origin sequence. In research this can be exploited by providing a repair template with a transgene of interest, flanked by homology arms that match the target DNA cut site. Adapted from: (Seol et al., 2018) with information from: (A. Ran et al., 2013; Seol et al., 2018)

In eukaryotes, Cas9 needs to enter the nucleus first to access the DNA. Fusion of an NLS site to both ends of the protein improves the transport inside and thus cleaving efficiency (P. Hu et al., 2018).

Cas9 specificity and safety

When using genome editing tools, specificity is always a major concern. This is true for pre-clinical- and especially for clinical research (Carlaw et al., 2020). Off-target cleavage may affect a patient in a potentially extensive way up to chromosomal sequence disruptions and translocations (Q. Wang et al., 2021). The same can happen on-target, but as the site is specifically chosen, the potential outcome can be predicted and managed to a certain extent (Bothmer et al., 2020). Undesired cleavage is not uncommon in CRISPR/Cas systems. Cas9 executes its nuclease ability even with several mismatches between guide RNA and target, especially those distant to the PAM sequence. Furthermore, even with slight differences in PAM, the target can still be recognized (Q. Wang et al., 2021). In the context of its biological derived function this can be beneficial for the organism (Westra et al., 2016) but needs to be prevented in science (Carlaw et al., 2020). Several isoforms of SpCas9 have been developed over the years to address this topic. By reducing the binding affinity of Cas9 to the target DNA, less mismatches are tolerated, as the guide RNA connection has to compensate. Other variants modify the cleavage process. Nickases for example only introduce single strand breaks, which alone can be sufficient for

HDR. A DSB in this case is only achieved if a second complex binds and cuts the other strand. Nicks can be efficiently repaired, thus reducing problematic off-target effects. Nuclease-inactive dCas9 does not cut the target at all and can be fused to a variety of effector domains to carry out tasks like transcription repression or activation (Tycko et al., 2016).

Unfortunately, higher specificity is usually paid by lower activity. In this project we decided to go with SpCas9-HF1. Four mutations decrease binding affinity to the target DNA, thus less mismatches are tolerated within the guide RNA. The protein showed on-target efficiency of 70-85% of wild-type cas9 and off-targets were nearly undetectable (Kleinstiver et al., 2016; Schmid-Burgk et al., 2020).

Further thoughts on safety include possible RNA targeting and immunogenicity of the Cas protein. RNA targeting by Cas9 types has been reported by Strutt et al. and should therefore also be recognized as possible undesired outcome (Strutt et al., 2018). Besides off-target effects, immunogenicity is always a concern when going into the clinic. The two Cas9 types prominently used are derived from *Staphylococcus aureus* (SaCas9) and *Streptococcus pyogenes* (SpCas9). These organisms are abundant in the human community. 80% of healthy people show an existing immune response. However, this is primarily directed against externally accessible structures of the bacteria or secreted proteins. Since Cas9 is an intracellular protein, a high immunogenic relevance is not expected. Nevertheless, Cas9 specific antibodies and leucocytes were found after treatment in mice, concluding that Cas9 does indeed triggers both a cellular and humoral immune response. On top of that, investigations of human serum showed pre-existing T-cells and antibodies against SaCas9 and SpCas9. Immunity against Cas9 may lead to adverse immune responses and reduced efficiency of an intended treatment as well as a reduced durability with cells expressing Cas9 being removed (Mehta & Merkel, 2020).

Cas9 in clinic

When looking at current clinical trials there are more than 30 related to CRISPR/Cas9, most of them in recruiting phase (NLM, retrieved 2022). Interventions can be split in *ex vivo* and *in vivo*. In ***ex vivo*** studies, cells of the patients are isolated, modified, and reinfused. This is of special interest in the context of HIV infection and cancer (Hirakawa et al., 2020). In HIV infection CCR5 is the key coreceptor for viral entry. Naturally occurring CCR5 mutations can eradicate the virus in HSPC transplant

recipients. CRISPR-mediated CCR5 ablation may be rewarded with the same outcome. Unfortunately, CCR5 ablation rates in bone marrow samples are varying and did lead to significant change in persistent infection. Still, long time engraftment of edited cells was achieved, proofing the principle (L. Xu et al., 2019). In cancer therapy, the patients' T-Cells can be modified with a chimeric antigen receptor (CAR) to be specific for tumour cells and through CRISPR/Cas9 mediated gene knockout enhanced for activation (Trial Identifier: NCT03747965) (Hirakawa et al., 2020). Successful results have also been obtained for the treatment of β-thalassemia (TDT) and sickle cell disease, monogenic, potentially life-threatening diseases that lead to inefficient erythrocytes (Frangoul et al., 2021).

There was also one *in vivo* study planned to treat persistent HPV infection, the outcome however is unknown (NCT03057912) (Z. Hu et al., 2014). Most notable of course was the incidence of the "CRISPR Babies". He Jiankui used the CRISPR system to knock out CCR5 in human embryos, subsequently implanted into their mothers and born. The CCR5 knockout was believed to protect them from HIV infection. It was germ line editing, meaning the mutation is not only present in the edited humans but also in their offspring. Naturally, He's research sparked an enormous global outcry and ethical concerns. Noteworthy are additional factors: (i) mutation in CCR5 is not going to protect 100% from HIV infection as CXCR4 can also serve as coreceptor, (ii) one of the twins seems to be heterozygote, thus one allele still expresses CCR5, though possibly at lower levels and (iii), the research can hardly be described as a last resort lifesaving effort as there are other ways to prevent and treat HIV infection (Greely, 2019).

1.3.2 Cas13d: Adaption, properties, and safety

Adaption in science

Cas13d belongs to class II type VI of CRISPR/Cas systems. Like other representatives of this type, it is an RNA dependent RNA targeting multifunctional protein. Type VI systems characterized beforehand have already been successfully used in the laboratory. However, their large size limits delivery via vectors. Cas13d is significantly smaller in size, being about 930 amino acids long compared to other Cas13 subtypes ranging in the area of 1200 amino acids. As noted above, Cas13d is a multifunctional protein that processes its array without any necessary cofactors. It can be directed by either suppling an array with multiple guide spacers at once or by expressing processed guide RNAs directly. In an array a 30nt spacer is flanked by two 36nt long direct repeats. During processing, Cas13d cuts away 6 bases of the direct repeat 5' and 8 bases of the spacer 3', therefore a processed guide consists of a 30nt repeat and 22nt spacer. Spacer design does not require any PAM or PFS motif. The cleavage position is not based on any obvious rule, however, there seems to be a bias towards uracil's. Other sequence compositions or motifs may also play a role (Konermann et al., 2018). Another highlight of Cas13d is its high knockdown efficiency and specificity. Investigations by Konermann et al. of Cas13d derived from *Ruminococcus flavefaciens* XPD3002, hereinafter referred to as CasRx, showed knockdown rates of 96% compared to 65% using RNAi *in vitro*. Fusion to NLS signals was beneficial (Konermann et al., 2018).

Off-target effects, well-known in high rates for RNAi, where undetectable for CasRx. Possible bystander cleavage, being of subordinate property in cells, seems not to lead to any off-targets either. These properties - high efficiency, and specificity - make CasRx an interesting alternative to RNAi based methods (Konermann et al., 2018). High specificity is also reported for other Cas13 subtypes (Abudayyeh et al., 2017) but off-targets are nevertheless not unheard of and therefore still need to be taken into account (Buchman et al., 2020).

What happens after mRNA cleavage?

When mRNA is targeted with the purpose of knocking down a gene's protein translation, the question, what happens after cleavage, arises. For this, one needs to look into the production, processing, and

degradation of mRNA. In short, mRNA is the intermediate between DNA and protein. In eukaryotes, mRNA is produced by RNA polymerase II, which reads the antisense DNA strand to produce a sense product, relative to the direction of the genes' coding sequence. Eukaryotes have three RNA polymerases, I – III, in contrast to prokaryotes with only one type. Each polymerase is responsible for a specific set of RNAs. Polymerase I produces ribosomal RNAs, II mRNA and RNAs involved in gene regulation such as micro RNAs, and III transfer RNAs and other short RNAs. Transcription starts at the promoter, the initiation sequence on the DNA, and stops at the terminator (Alberts et al., 2015). Initiation is highly similar across polymerase types, but termination relies on different mechanisms. Pol I and II make use of additional proteins, with Pol II having the more complex interplay. Pol II on the other hand, relies on a physically conveyed separation of the connection between polymerase and DNA, mediated by structural characteristics in the produced RNA (Arimbasseri et al., 2013). What follows is capping, the addition of a modified guanine nucleotide that protects the mRNA from 5'-3' exonuclease degradation and being important for nuclear export and translation initiation. Subsequent splicing removes noncoding introns and fuses together exons, the coding regions of the mature mRNA. Finally, polyadenylation stabilizes the 3' end of the mRNA. As with the capping, the polyadenylation is important for export, translation and protection from exonuclease activity (Alberts et al., 2015) Many viruses, such as adenovirus, rely on the same host cell mechanics (Bouvet et al., 2012; L. Hwang et al., 1998; X. Li & Palese, 1994; H. Zhao et al., 2014).

Cleavage in the middle of an mRNA yields two RNA strands, whereas one is missing the cap and the other the poly A tail. Thus, both are rapidly degraded by exonucleases, in 5'-> 3' direction or vice versa (Alberts et al., 2015). Exonuclease activity, responsible for RNA degradation is usually attributed to the cytoplasm but nevertheless, is also found within the nucleus. Transcripts, unable to be exported, would otherwise accumulate pathologically (Das et al., 2003; H. Liu et al., 2014).

Knockdown or knockout?

Choosing knockdown over a knockout approach can have advantages. First of all, a knockdown is reversible as no permanent changes in DNA are introduced (Boettcher & McManus, 2015). Therefore, cells artificially expressing Cas13d will stop doing so over time, depending on their turnover, when a non-integrating vector was used for delivery (C. S. Lee et al., 2017). Genetic changes in the DNA, made by CRISPR/Cas9 e.g. are permanent and are transmitted to daughter cells even after expression of CRISPR/Cas9 stopped. Thus, genetic disorders can be potentially cured with a single treatment, but

possible off-target effects will have greater impact (Boettcher & McManus, 2015; F. A. Ran et al., 2015). Furthermore, a knockdown does not rely on the difficult to predict outcome of DNA modification, introduction of frameshift mutations and pre mature stop codons e.g. (Boettcher & McManus, 2015).

Cas13d in Clinic

Due to the very recent description (Konermann et al., 2018), CasRx has not yet found widespread use or clinical application (NLM, retrieved 2022). Nevertheless, there is an *in vivo* study in the animal system, showing efficient mRNA knockdown by CasRx (Kushawah et al., 2020) and the use of Cas13 and Cas13d in particular has been proposed as possible treatment option for the novel SARS-CoV-2 (Lotfi & Rezaei, 2020; Nguyen et al., 2020).

1.4 RNA interference

1.4.1 Principle

RNA interference (RNAi) was first discovered in *C. elegans* and was since then identified as a general conserved pathway in organisms. Main mediators are small interfering RNAs (siRNAs) and micro RNAs (miRNAs) which silence gene expression in sequence specific manner by promoting degradation of complementary mRNA. Both types are loaded into the same protein complex, carrying out the effector function, but there are differences. MiRNAs are expressed from the genome and while siRNAs may also be of internal origin, they are usually from viral- or other exogenous sources (Carthew & Sontheimer, 2009). SiRNAs target mRNA by highly specific sequence base paring up to a perfect match and lead to cleavage of the binding partner. MiRNA does not require high homology and also imperfect pairing leads to robust transcriptional inhibition and subsequent degradation of target mRNA. Thus, siRNAs regulate expression of one gene, whereas miRNAs can regulate that of various genes with mRNAs sharing short homologies (Lam et al., 2015).

At the beginning of the RNAi pathway is double stranded RNA (dsRNA). In the siRNA process the dsRNA, as noted above usually of exogenous origin, is processed by the RNase III-like enzyme DICER into short 21-23 nucleotide long dsRNA molecules, the siRNAs. They subsequently interact and activate the RNA-induced silencing complex (RISC). The endonuclease argonaute 2 (AGO2), part of RISC, degrades the passenger strand, the sense strand of the siRNA, and only the guide strand (antisense) remains associated with the complex. This strand then guides the complex to its matching mRNA for subsequent cleavage by AGO2 Figure 19 (Lam et al., 2015).
MiRNA is expressed from the genome by RNA polymerase II resulting in a capped and polyadenylated primary-miRNA (pri-miRNA). The pri-miRNA consists of a stem loop, including a double-stranded stretch and single stranded overhangs. Within the nucleus the pri-miRNA is processed by the microprocessor complex into the precursor miRNA (pre-miRNA). The complex consists of DGCR8, a protein with two RNA binding domains, and Drosha, like DICER belonging to the family of RNase III-like enzymes. The pre-miRNA is exported to the cytoplasm by Exportin-5 and RanGTP. As is the case with siRNA, the RNA molecule is further processed by DICER and loaded into RISC where the passenger strand is discarded. Imperfect base pairing of miRNA within the RISC to its target, leads to subsequent transcription repression, or degradation via deadenylation Figure 19 (Wilson & Doudna, 2013).

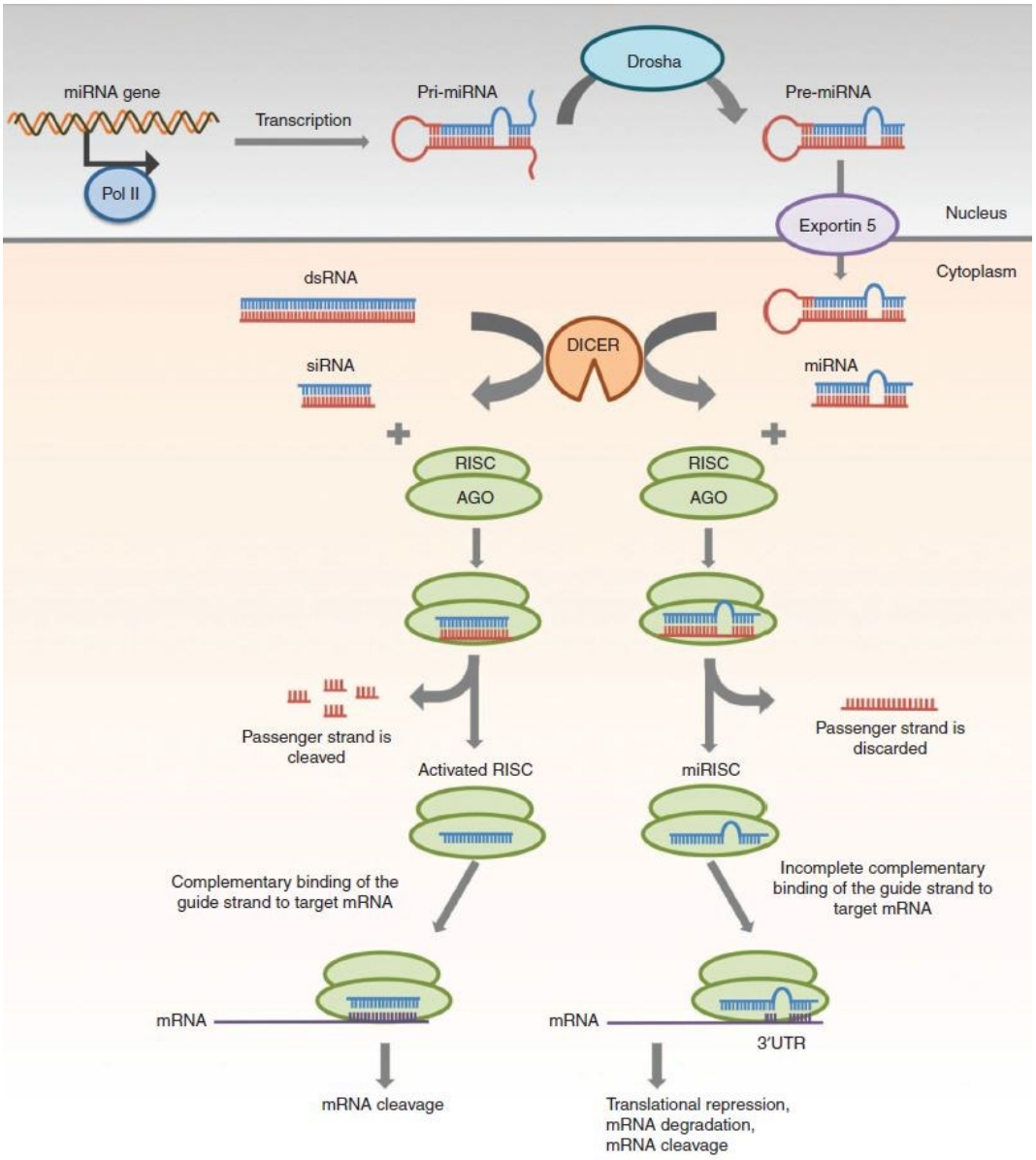


Figure 19 **RNA interference (RNAi) pathway**. RNAi is a conserved pathway for transcriptional regulation of gene expression. **Left**: An siRNA is generated from double-stranded RNA by DICER and subsequently loaded into RISC complex. There, AGO cleaves the passenger strand and the guide strand remains. The complex binds to target mRNA with high sequence specificity and cleaves the transcript. **Right**: Contrary to siRNAs whose origin is usually exogenous, miRNAs are transcribed from the genome. Within the nucleolus the thus expressed pri-miRNA is processed by Drosha, generating pre-miRNA. It is then transported to the cytoplasm by Exportin-5 and further processed by DICER. After incorporation into RISC, the passenger strand is discarded. Interference of miRNA relies on incomplete base paring; thus a greater set of different mRNAs can be targeted, via transcriptional repression and degradation by deadenylation and in rare cases, similar to siRNAs, cleavage. Adapted from: (Lam et al., 2015)

In humans there are four **argonaute proteins** (1-4). They are the central effector proteins that mediate interference. Only AGO2 has cleaving ability, but as noted above, cleaving of a target is not essential for transcription inhibition (Wilson & Doudna, 2013).

1.4.2 Adaption for Science

The advantage of using RNAi in science is clear. Since the RNAi pathway is highly conserved and is therefore present in many organisms and cell types, all that is required is the addition of artificially created siRNAs, miRNAs or short hairpin RNAs (shRNAs), to quickly suppress a large number of different genes, quickly and easily, in sequence specific manner (Agrawal et al., 2003). Possible application areas are abundant, reaching from reverse genetic approaches (Zhai et al., 2009) in basic research, to therapeutics in clinic (Bobbin & Rossi, 2016).

RNAi in science relies on different mediators. **SiRNAs**, short double-stranded RNAs, can be chemically synthesized and delivered by lipid nanoparticles (Tatiparti et al., 2017). **ShRNAs** are transcribed from transduced DNA within the cell. The necessary double-stranded character is achieved by the encoded hairpin in the middle of the transcript. Therefore, shRNAs can also be delivered by viral vectors, which leads to higher transduction efficiency, flexibility and the possibility of stable integration (Moore et al., 2010). ShRNAs make use of the miRNA pathway so far as they are expressed as pri-shRNA. Drosha processing leads to pre-shRNA, which is exported from the nucleus by Exportin 5. Further processing by DICER produces the final siRNA ready for loading into RISC (S. Singh et al., 2011). Artificial micro RNAs (**amiRNAs**) retain the backbone of miRNAs found in endogenous genes and are produced and processed equivalent. Their target sequence however, is modified to target an mRNA of interest (Mickiewicz et al., 2016). Like shRNAs, amiRNAs can also be expressed from viral vectors (Fu et al., 2019).

1.4.3 Specificity and Safety

As noted above siRNAs rely on highly specific base pairing and usually only have one target. In miRNA related interference, imperfect base pairing is enough for the knockdown to take place, thus multiple targets are possible (Lam et al., 2015). However, these modes of actions are not strictly divided. In the case of highly specific base paring by miRNA, an siRNA-like targeting type can occur (Lam et al., 2015), or in case of imperfect base pairing of siRNAs, miRNA-like off- (or multiple) targeting (Neumeier & Meister, 2020).

Off-targets due to sequence homologies can be reduced via **clever design**. Avoiding overall homologies to other RNAs, via the help of sequence databases and design tools, is the standard. Still,

predicting matches within the seed region (residues 2-7 from the 5' end) of the siRNA or miRNA is more difficult as they are abundantly found on various mRNAs. It is those seed region homologies that lead to significant off-target effects (Cullen, 2006; Neumeier & Meister, 2020). Besides design considerations, **modifications**, such as mild destabilization of the interaction between guide RNA and target, or **pooling** can additionally reduce undesired effects. In pooling, multiple RNAi-RNAs target the same mRNA. Off-target effects of an individual RNA are reduced as it is present in lower dose (Neumeier & Meister, 2020). Further obstacles, especially for RNAi based drugs, include: (i) immunogenic reactions to double-stranded RNA, (ii) inhibition of the endogenous RNAi pathway due to resource competition and (iii) dose limiting toxicity as a result of off-target effects (Cullen, 2006; Setten et al., 2019).

This shows how important subsequent testing and confirmation of the specificity of an RNAi system is. Whole transcriptome sequencing is an option e.g. Nevertheless, even with extensive testing, some undesired effects may still be impossible to prevent (Cullen, 2006; Neumeier & Meister, 2020; Setten et al., 2019).

1.4.4 RNAi in the clinic

RNAi and its mediators, siRNAs and miRNAs, are promising candidates for treatments. A multitude of different diseases is based on expression of undesired or mutated genes as well as overexpression of otherwise accurate genes. In contrast to small drug molecules or monoclonal antibody therapies, which can only target a limited set of proteins, RNAi can be used to knock down basically any gene there is (Lam et al., 2015). These characteristics lead to a spirit of optimism in multiple companies by 2003. Initial clinical trials using unmodified siRNAs did not live up to the expectations however, showing debatable benefit and immune side effects. Following trials, applying nano particle formulated siRNAs, displayed certain therapeutic effects but also significant dose-limiting toxicities and insufficient efficacy. By 2010 many big companies have left the field, with only smaller ones and academic institutes remaining. Besides setbacks, they steadily improved design, chemical composition, and delivery. Thus, safer and more efficient RNAi therapies are available, but challenges remain (Setten et al., 2019).

There are currently 12 active trials listed at clinicaltrials.gov when looking up the tag “RNAi” (NLM, retrieved 2022). Recent results showed successes ranging from eye drop solutions for dry eye disease to subcutaneous delivery for reduction of low density lipoprotein cholesterol and intravenous application against chronic hepatitis B infection (Setten et al., 2019).

1.5 CRISPR/Cas and RNAi in antiviral strategies

1.5.1 CRISPR/Cas

CRISPR/Cas is per definition an antiviral immune system, and defense against foreign genetic elements in general, therefore the application as antiviral system in humans is not farfetched. In principle, one only needs to provide a guide RNA targeting the virus genome or other virus-related genetic element. Several studies, *in vitro* and *in vivo*, addressing various virus types, did exactly that. Nevertheless, it is not as easy as it may seem, there are different strategies available and obstacles to overcome (Soppe & Lebbink, 2017).

In HIV infection two main ways are possible - targeting cellular factors or targeting the virus. As noted above, abolishing function of the two main host cell co-receptors CCR5 and CXCR4 impairs HIV spread. HIV is a single stranded positive-sense, enveloped RNA virus. It primarily infects T-cells, in whose genome it integrates via reverse transcription of its RNA genome into DNA. There it persists as latent pro-virus (Soppe & Lebbink, 2017). The RNA genome itself can be targeted by RNA cleaving CRISPR/Cas systems. Cas13a has shown promising results in destroying viral RNA, both genomic and transcripted from pro-virus integrations, leading to strong inhibition of infection (Yin et al., 2020). Reverse transcribed DNA intermediates can be targeted using Cas9. Studies report promising results in human cells, but major obstacle are arising escape mutants. The escape mutants are primarily directly arising through Cas9 cleavage itself. As NHEJ leads to a more or less random outcome, besides knockout- also silent mutations, without influence on the viral gene's function, are possible that prevent subsequent Cas9 targeting. These resistant escape mutants then replicate unhindered. This was observed to a high degree when targeting non-essential genes of the virus and to a lower when targeting essential genes. Conserved sequences in essential genes hardly tolerate indel mutations, still, viral escape was observed in all cases (G. Wang et al., 2016; Z. Wang et al., 2016). Multiplexing, combining more than one guide RNA, targeting multiple domains of the virus, can reduce emergence of escape mutants and in the best case prevent them all together (Darcis et al., 2019).

Hepatitis B virus has a partially double-stranded DNA genome and replicates in human hepatocytes. There, the viral genome is transported into the nucleus and converted into covalently closed circular DNA (cccDNA). DNA replication occurs via an RNA intermediate that is converted back to DNA by the

viral reverse transcriptase. There are drugs available that influence the host immune response or inhibit the reverse transcription step. The cccDNA however remains within the nucleus and servers as persistent template for transcription. Treatments therefore need to be long-term and may lead to resistances. Targeting cccDNA by RNA guided nucleases such as CRISPR/Cas is therefore a promising strategy (X. Liu et al., 2015). Indeed, there are several studies that reported high rates of inhibition by using CRISPR/Cas9 to target the cccDNA. Inhibition was further increased through multiplexing (Kennedy et al., 2015; X. Liu et al., 2015; Schiwon et al., 2018; Seeger & Sohn, 2014).

Further successful examples of antiviral CRISPR/Cas9 application include elimination or inhibition of: polyoma virus JC (Chou et al., 2016; Wollebo et al., 2015), vaccinia virus as a model of orthopoxviruses (Siegrist et al., 2020), human cytomegalovirus (Gergen et al., 2018) and the African swine fever virus (Hubner et al., 2018).

1.5.2 RNAi

The role RNAi has in modulating gene expression on the mRNA level can be made use of as an antiviral strategy. In plants, fungi, and invertebrate's antiviral immunity via RNAi is an endogenous function, processing viral dsRNA into siRNAs and subsequently mounting a strong antiviral defense. In mammals, even though they have the same conserved mechanism, it was less clear. In mammals the interferon pathway, activated by long dsRNA, seems to be the first line of defense against viral RNA. Furthermore, activation of the interferon pathway represses RNAi, pointing to possible competition between the two (Levanova & Poranen, 2018; Schuster et al., 2019). Still recent studies found viral derived siRNAs in mammalian cells infected with various viruses. That hints at an endogenous, interferon independent, antiviral function also in mammals (Ding et al., 2018; Schuster et al., 2019).

Regardless of natural endogenous functions, it is clear that artificially introduced siRNAs and miRNAs can be used to repress viral replication. Injection of siRNA, targeted against conserved regions of hepatitis B virus mRNA, greatly and stably reduced levels of transcripts, proteins and DNA (Wooddell et al., 2013). The same was shown for two artificial miRNAs directed against conserved genes of west nile virus. The reduction in virus titer was as high as 97,11%, 96 hours post infection (Karothia et al., 2020). In nonhuman primates, lipid nanoparticle enclosed siRNAs where able to provide protection from ebola virus. Even though treated animals displayed symptoms of disseminated disease, the

course was less serious and concluded in full recovery. Untreated animals eventually died (Thi et al., 2015).

In adenovirus, siRNAs were used *in vitro* to target a set of different viral genes, directly or indirectly involved in viral DNA replication. The aim was to find out which gene is best used for virus inhibition. It turned out that early genes are the most suitable candidates compared to late genes. A siRNA directed against the viral polymerase reduced the genome copy number by several orders of magnitude (Kneidinger et al., 2012). The same picture was also shown using miRNAs *in vitro*. Investigating a set of miRNAs targeted against key adenovirus genes, the one against pTP showed highest inhibition rates. Reduction rates were as high as 2.6 orders of magnitude by using six repetitive copies of miRNA sequences. The combination with cidofovir further increased reduction rates (Ibrisimovic et al., 2013). The use of miRNAs against adenovirus infection was later confirmed *in vivo* in the Syrian hamster model with reduction rates of up to 2 orders of magnitude and reduction of adenovirus related liver damage (Schaar et al., 2017).

2 Aim and benefit of the thesis

The aim of the thesis is to find new ways of treating severe viral infections, with human adenovirus as model, which remain a problem to this day. While diagnostic procedures and drug development have seen improvement in recent years the challenge remains. Especially in immune suppressed patients, human adenovirus leads to high mortality rates. Limited treatment options demand the development of new strategies (Alvarez-Cardona et al., 2020; Lion, 2019; Lynch et al., 2011).

The research conducted in this thesis shall highlight the possibilities of targeting adenoviral infection and shall serve as proof of principle and advanced foundation for future projects, with the common goal to move ever closer to a clinical applicable therapy.

The following **main questions and objectives** are addressed:

- Can CRISPR/Cas be applied to target adenovirus and its replication, leading to a reduction of overall viral load?

- What are the inhibition efficiencies when using RNA targeting CRISPR/CasRx and DNA targeting CIRSPR/Cas9?

- Development and step-by-step optimization of a CRISPR/Cas based therapy platform for adenoviral infections.
- Continued investigation of CRISPR/Cas as antiviral therapy.
- Providing novel insights and data on RNA targeting CRISPR/Cas as antiviral therapy against a highly replicative human DNA virus.
- Identification of ways to combine CRISPR/Cas and amiRNA mediated RNAi in the context of inhibition of adenoviral replication.

Summarized, the overall goal is to point out novel treatment options of adenoviral infections and to contribute to CRISPR/Cas research, thus, paving the way for future CRISPR therapeutics and their antiviral application to improve patient welfare.

3 Thesis Topics

3.1 Overview of thesis topics and author's contribution

The following chapter consists of three topics:

The **first topic** shows the development and evaluation of a step-by-step optimized CRISPR/CasRx expression system, delivered to cells by recombinant adenovirus vectors, to inhibit wild-type human adenovirus replication *in vitro*, by targeting viral *pol* and *pTP* mRNAs.

The **second topic** studies ways of combining CRISPR/CasRx with RNAi, with multiple different types of multiplexed vectors generated, to identify the most efficient design.

The **third topic** evaluates the efficacy of a CRISPR/Cas9 expression system to inhibit wild-type human adenovirus replication *in vitro*, by targeting the viral gene E1A. Furthermore, ways of multiplexing guide RNAs and combinations with RNAi are investigated and sequencing results evaluated for Cas9 targeting efficiency and specificity.

As of writing these words, the manuscript of the first topic is posed for submission in a peer-reviewed journal and the manuscript of the third topic is accepted in the peer-reviewed journal: Molecular Therapies - Nucleic Acids.

I hereby declare that I have significantly contributed to the realization of the studies presented in this thesis.

In the first topic I was actively involved in study- and assay design as well as in the overall study concept. I did the majority of vector construction and experimental work of Fig.1-Fig.8 and Fig.S1-Fig.S3. Furthermore, I contributed the majority of data evaluation and interpretation as well as figure design and manuscript writing.

In the second topic I was actively involved in study- and assay design as well as in the overall topic concept. I constructed the vectors and did the experimental work of Fig.1 and Fig.2. Furthermore, I contributed the majority of data evaluation and interpretation as well as figure design and writing.

In the third topic I was actively involved in study- and assay design as well as in evaluation and interpretation of results and manuscript writing. Furthermore, I contributed to the design and generation of multiple vectors and viruses, applied in the study. I did the majority of vector production and experimental work of Fig.6 and Fig.7 and prepared samples for sequencing, evaluated in Fig.8.

Ing. Florian Reithofer, MSc
Krems, 01.01.2023

3.2 Manuscript: Inhibition of viral replication by RNA-Targeting Type VI-D CRISPR Effector CasRx

Inhibition of viral replication by RNA-Targeting Type VI-D CRISPR Effector CasRx

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One sentence summary:

Evaluation of characteristics of the RNA-targeting type VI-D CRISPR Effector CasRx as means of antiviral defence against the human pathogenic DNA virus adenovirus in a step-by-step optimized system.

Key Words:

Antiviral defence, viral replication inhibition, Adenovirus, CRISPR/Cas, CasRx, Cas13d, RNA-targeting, recombinant adenovirus, DNA virus.

Abstract

Viruses remain one of the major challenges to the community. Finding new and efficient ways of treating infections are a constant medical need, both on local and global scale. While this is true for the general population, it is of particular importance in high-risk individuals such as immunocompromised patients. Here, viruses, commonly believed to be harmless, pose a significant and life-threatening risk. Human adenovirus (HAdV), a non-enveloped DNA virus, is one of these. While being usually self-limiting in immune competent hosts, high mortality rates are reported in immunocompromised patients. Available treatments are limited and unsatisfactory.

Following reported data of RNA interference and its ability to inhibit adenoviral replication as well as advances in using CRISPR/Cas9 as antiviral agent, we applied the RNA-targeting Type VI-D CRISPR Effector CasRx to target HAdV-5 *pol* and *pTP* mRNAs, early expressed during the infection cycle and essential for replication. We develop a step-by-step optimized CRISPR/CasRx expression system, delivered to cells by recombinant adenovirus vectors, to inhibit HAdV-5 replication *in vitro*. Inhibition rates of HAdV-5 infectious units of up to 2 orders of magnitude in prophylactic- and of up to 1,5 orders of magnitude in therapeutic application underline the potential CRISPR/CasRx has as a promising candidate for targeting viral infections.

Introduction

Throughout history viral infections have caused a constant health risk to the human population. While extensive successes in understanding and treatment of viral diseases were made, they remain a major challenge (Oldstone, 2020). Previous and ongoing pandemics and their huge social and economic impact, highlight the constant need for new ways of targeting viral infections (Khan et al., 2020; Morens et al., 2020; Shang et al., 2021). However, pandemics, affecting communities on global scale, are not the only concern. Looking at subpopulations, immunocompromised individuals have a particular risk of getting life threatening viral diseases. Infections that would be usually cleared away in immune competent hosts, can have a serious impact on patients that are immunosuppressed. Disseminated diseases and high mortality rates are reported (Ahmad et al., 2021; Clausen & Zaffiri, 2020; Cukuranovic et al., 2012; Kotton & Fishman, 2005; Kraft et al., 2012). Treatments requiring immunosuppression are on the rise, with allogenic stem cell- or organ transplants as prominent examples, and so is the requirement of managing viral infections in this thus growing patient population (Lion, 2014).

The human adenovirus is one of many viral agents of concern to be noted here. Human adenoviruses, belonging to the *Adenoviridae* family, are medium-sized non-enveloped DNA viruses with a linear double stranded genome (Echavarria, 2008). They are classified into seven species A-G and a multitude of types (Ismail et al., 2018). Different tissue tropisms are reported for individual species. Species C representatives are of central concern in adenovirus related human pathologies. They account for more than half of adenoviral infections in immunosuppressed patients and represent a major percentage in immune competent hosts as well. They affect the respiratory and gastrointestinal tracts (Dhingra et al., 2019; Ghebremedhin, 2014). The first contact with adenoviruses mostly occurs during childhood. Symptoms are usually common cold like and self-limiting. A healthy immune system can quickly and efficiently clear away the virus which often leads to lifelong immunity. In immunocompromised patients however, adenovirus can cause life threatening disseminated diseases (Lynch et al., 2011).

Unfortunately, available treatment methods are limited and unsatisfactory. Cidofovir, a nucleoside-analog often used in severe cases, specifically binds and blocks viral DNA polymerase activity. However, the drug's potential is diminished by severe nephrotoxicity and the need for a robust immune system to eradicate the last traces of the virus. (Chamberlain et al., 2019; Hitchcock et al., 1996; Lion, 2019). Improvements were reported using Brincidofovir, prodrug of Cidofovir. This lipid-linked derivative showed improved bioavailability properties and safety profile, yet toxicity remains a factor (Londeree et al., 2020). Besides small molecule-based antivirals, antiviral therapy research increasingly employed methods such as RNA interference (RNAi) and the programmable nuclease Cas9 (Levanova & Poranen, 2018; Soppe & Lebbink, 2017). RNAi, mediated through small interfering RNAs (siRNAs) and artificial micro RNAs (amiRNAs), has shown to greatly reduce virus derived mRNA leading to a strong decrease in total viral load. These methods were reported successful for adenovirus and a variety of

other viruses and have since found their way into clinical trials (Ibrisimovic et al., 2013; Karothia et al., 2020; Kneidinger et al., 2012; Levanova & Poranen, 2018; Song et al., 2010; Thi et al., 2015; C. I. Wooddell et al., 2013). CRISPR/Cas9, revolutionary gene editing method (Strzyz, 2020), has been evaluated in the context of various viral infections as well. The quick and simple programmable character and its evolutionary purpose as defense against invading genetic elements underline the potential in treating viral infections. Direct targeting of a virus DNA genome or integrated DNA stage significantly reduced viral titer across different species. While reported results are primarily preclinical in nature, they nevertheless provide compelling support for CRISPR/Cas9 as an viable platform for treating viral infections (Soppe & Lebbink, 2017). Current clinical trials, in which Cas9 is applied as antiviral defense, focus on modifying cells to make them more resistant to viral infection: NCT03164135 (Xu et al., 2019), or to target a virus latent stage: NCT03057912 (D. Yin et al., 2021).

However, the question of off-target effects remains in CRISPR/Cas9 and RNAi (Barrangou et al., 2015). Furthermore, CRISPR/Cas9 has been shown to create its own escape mutants, resistant to renewed attack (Presloid & Novella, 2015; Z. Wang et al., 2016). Therefore, in this study, we combined aspects of both approaches by applying cutting edge RNA targeting CRISPR/Cas systems.

RNA targeting Cas proteins, have been known for quite some time and even Cas9 was attested limited ability of targeting RNA (O'Connell et al., 2014; A. A. Price et al., 2015; Zhu et al., 2018). Recently described CasRx enzyme, a Cas13d type of *Ruminococcus flavifaciens* XPD3002, showed high levels of specificity and knockdown efficiency, with previously limiting factors such as effector size, target site requirements and off-target activity improved (Burmistrz et al., 2020; Konermann et al., 2018).

In the following study we evaluated the potential of CRISPR/CasRx to inhibit adenoviral replication *in vitro*. As model target, we chose human adenovirus 5 (HAdV-5). For the delivery of the CRISPR system to the cells we used viral vectors, commonly used for delivery of expression cassettes (De Haan et al., 2021; Luther et al., 2018). We generated a series of replication incompetent HAdV-5 vectors, comprising our proposed antiviral platform. As the delivery vector is based on the same adenovirus type as the target virus, we expected the otherwise replication incompetent recombinant vector to be mobilized in cells simultaneously infected with the wild-type virus by complementation of the recombinant's missing genes. Furthermore, shared tissue tropism and competition for equal resources may pose additional benefit. Each vector contained the CasRx effector protein, and one gRNA, directed against an essential gene of the virus, the viral polymerase (*pol*) or the preterminal protein (*pTP*). Both are expressed early in the infection cycle and have a direct effect on viral genome replication: the polymerase replicates the DNA and pTP serves as anchor, attached to the DNA ends. In a later step, pTP is processed and thus presented in its final form, the terminal protein (TP) necessary in genome stability and packaging (Charman et al., 2019).

Material and Methods

Cell culture

A549 (human epithelial lung carcinoma; ATCC CRL-185) and HEK293 (human embryonic kidney; ATCC CRL-1573) were cultured in Dulbecco's Modified Eagles Medium (DMEM), modified with high glucose and GlutaMAX, supplemented with 10% heat inactivated fetal bovine serum (Thermo Fischer Scientific,Vienna, Austria). T-REx™-293, modified HEK293, stably expressing the tetracycline repressor protein (Thermo Fisher Scientific, Vienna, Austria), were cultured in the same medium but supplemented with certified tetracycline-free 10% heat inactivated fetal bovine serum (FBS, Takara Bio Europe, Paris, France) and Blasticidin at a concentration of 10µg/mL. All cell lines were kept in a humidified 5% CO_2 atmosphere at 37 °C. Cell health was closely monitored and ensured to be at high level at all times and no observable differences to be present in assays across samples and controls.

Spacer design and plasmid construction

Choice of target location within the viral genes *pTP* and *pol* initially relied on published and unpublished data by Ibrisimovic et al. and Kneidinger et al. which evaluated the site's receptibility for RNAi based knockdown (Ibrisimovic et al., 2013; Kneidinger et al., 2012). Sequences of a non-targeting control spacer, the gRNA backbone and the coding sequence of CasRx were adapted from Konermann et al. (Konermann et al., 2018). To reduce the risk of off-target effects via shared homologies, each spacer sequence was checked against the RefSeq human transcriptome using the online tools TagScan and GGGenome (accessible online at https://gggenome.dbcls.jp/ and https://ccg.epfl.ch/ , 2019). To secure specificity and reduce possible gRNA off-targets, filter in tools was set to maximum number of 2 to 3 mismatched base-pairs excluding all potential gRNAs with higher number of target mismatches. In addition, the non-targeting control was tested with BLAST engine against the sequence collection of *Adenoviridae* using standard parameters including adjustment for short sequences. Construction of recombinant adenovirus delivery particles relied on the Gateway™ system, involving assembly of all functional parts in the entry plasmid pENTR4 (Thermo Fisher Scientific, Vienna, Austria) and subsequent recombination with the destination vector pAd/PL-DEST (Thermo Fisher, Vienna, Austria), supplying the viral backbone. Thus, the assembled cassette is transferred to the *E1A* deleted region of the adenoviral based destination vector which is subsequently used to generate first generation recombinant adenovirus delivery vehicles.

Overall, three different platforms were designed. The initial proof of principle system consisted of the 2V-CasRx-EGFP vector that could be easily combined with different gRNA expression vectors (eg. 2V-pTP-gRNA1). The CasRx expression plasmid was generated by amplifying the sequence of a constitutive active CMV promoter/enhancer complex followed by two $TetO_2$ tetracycline repressor binding sites by

PCR from vector AdTO-ptp-mi5, designed earlier in our lab (Ibrisimovic et al., 2013), with primers F_pENT4_CHF_CRX_NB 5'-TGCAGGATGACCGGTCATC-3' and R_pENT4_CHF_CRX_NB 5'-GGTGGCGGCTCTCCCTATA-3'.

The CasRx sequence, together with supporting modules, nuclear localisation signals, T2A ribosome skipping site, and EGFP was amplified from EF1A-CasRx-2A-EGFP-WPRE (Konermann et al., 2018) with primers F_Ins_CRX_NB 5'-GGGAGAGCCGCCACCATGAGCCCCAAGAAGAAGAG-3' R_Ins_CRX_NB 5'-ACCGGTCATCCTGCACGGTATCGATGCGGGGAG-3'. The assembly of promoter- and CasRx fragments was done using the NEBuilder protocol (New England Biolabs, Frankfurt am Main, Germany). In gRNA expressing plasmids, an empty pENTR4 served as backbone while the gRNA cassette was designed according to Konermann et al. 2018. Sequence oligos were generated by gene synthesis (Biomatik, Delaware, USA) and were cloned via *NcoI* and *NotI* sites of vector pENTR4. **(Fig. 1-A).**

All-in-one platform plasmids (eg. 1V-pTP-gRNA1) were constructed by first extending gRNA cassettes by restriction sites *AscI* and *XbaI* with primers F_AscI_CrxgRNA_amp 5'-CATGGCGCGCCGAGGGCCTATTT-3' and R_XbaI_CrxgRNA_amp 5'-GAATCTAGAGGTACCATGCGGTAACCGCG-3'. 2V-CasRx-EGFP vector served as backbone in which the gRNA was cloned into via *AscI/XbaI*-restriction digest and subsequent ligation with T4 ligase according to supplied protocol, followed by steps explained in 0 (New England Biolabs, Frankfurt am Main, Germany) **(Fig. 1-B).**

To generate vectors NT-Tet-gRNA and Pol-Tet-gRNA2, vectors 1V-NT-gRNA and 1V-Pol-gRNA2 were adapted by adding two $TetO_2$ sites to their U6 promotors using the Q5 site directed mutagenesis kit (New England Biolabs, Frankfurt am Main, Germany) with primers Change_TetU6_F 5'-atatctccctatcagtgatagagACCGAACCCCTACCAACT-3' and Change_TetU6_R 5'-ataatctctatcactgatagggagTTTCAAGTTACGGTAAGCATATG-3'. Putting gRNAs, besides CasRx, under $TetO_2$ is expected to further reduce possible self-targeting in packaging cells and thus prevent complications in recombinant virus amplification **(Fig. 1-C).**

Virus amplification, confirmation of genotype and determination of infectious viral particle number

Final versions of pENTR4 entry plasmids were recombined with pAd/PL/Dest destination plasmids according to the Gateway system protocol (Thermo Fisher Scientific, Vienna, Austria). The resulting adenoviral vectors were subsequently digested with *PacI* to expose the inverted terminal repeats (ITRs). Due to the linearized DNA's large size of over 30 kb, the purification was done by phenol chloroform extraction and subsequent EtOH precipitation using standard protocols. 700ng of purified restricted destination vector were diluted in 50µL OptiMem (Gibco™) and mixed together with 1,5µL Lipofectamin 2000 (Thermo Fisher Scientific, Vienna, Austria). The mix was used to transfect 1,8e + 05 T-REx™-293, supplementing viral *E1A* gene, missing in recombinant adenoviruses and the tetracycline repressor protein. As soon as cytopathic effect became apparent and around 80% of cells detached, the cells were

harvested, resuspended in 2 to 3 mL fresh medium and subjected to three consecutive freeze thaw cycles causing the cell's lysis. Virus was purified using the "Fast Trap Adenovirus Purification and Concentration Kit" according to the manufacturer (Merck, Darmstadt, Germany).

Determining viral titers in stocks as well as in assays was done using the AdenoX Rapid Titer Kit (Takara Bio Europe, Paris, France) according to protocol but with antibody dilutions doubled. Recombinant virus was titered on T-REx™-293 cells and wild-type HAdV-5 on A549 cells. Prior to application in experiments, DNA was extracted from each stock by the DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and the corresponding area of interest, amplified by PCR, sent for sequencing (Microsynth, Balgach, Switzerland) to confirm the correct genotype.

Amplification and subsequent extraction of circular plasmid DNA

30µL of electro-competent NEB-10β cells (New England Biolabs, Frankfurt am Main, Germany) were thawed on ice for 10 minutes and up to 3µL of the ligation reaction was added. The mixture was transferred to ice cold electro poration cuvettes with 0,1 cm gap and pulsed using 2 kV at a MicroPulse Electroporator (Bio-Rad, Hercules, CA,USA). Immediately after, 970µL prewarmed outgrowth medium was added, and the cells were incubated for 1 hour at 37°C. Afterwards, the cells were plated on LB-agar with corresponding selection antibiotic added. The next day, colonies were picked into 20µL of nuclease free water and checked by colony PCR using OneTaq® 2X Master Mix (New England Biolabs, Frankfurt am Main, Germany). Clones showing the expected bands in gel electrophoresis were amplified overnight in antibiotic-supplemented liquid broth (LB) medium and cells harvested the following day. Depending on culture volume, circular DNA was extracted either by the FavorPrep Plasmid Extraction Mini Kit (Favorgen, Vienna, Austria) or by the QIA Filter Plasmid Midi Kit (QIAGEN, Hilden, Germany) according to supplied protocols. The extracted plasmids were confirmed by restriction digest and consecutive sequencing (Microsynth, Balgach, Switzerland).

Wild-type adenovirus inhibition assays

Treatment was performed via two approaches, prophylactic and therapeutical. Under prophylactic conditions 1,5e + 04 A549 cells, diluted in 100µL medium, were seeded into the wells of 96-well plates and directly transduced with recombinant adenovirus. The all-in-one recombinant viruses (1V) were applied at an MOI of 50 $TCID_{50}$. In the two-component system (2V) the MOI of CasRx and gRNA viruses was increased to 100 $TCID_{50}$. Applying the constructs that harbour $TetO_2$-regulated gRNAs, an MOI of 100 $TCID_{50}$ was used as well. Cell viability and transduction efficiency across samples can be seen in Figure S1. 48 hours later, cells were infected with wild-type HAdV-5, diluted in 50µL medium, at an MOI of 0,01 $TCID_{50}$. In prophylactic conditions a total of four plates was prepared and subsequently harvested by three consecutive freeze thaw cycles at zero-, two-, four- and six- days post wild-type infection.

Under therapeutical conditions 1,5 e + 04 A549 cells, diluted in 100µl medium, were seeded into the wells of 96-well plates and reverse infected with wild-type adenovirus, diluted in 50µL medium, at an MOI of 0,01 $TCID_{50}$. After six hours incubation, cells were transduced with recombinant adenovirus at MOI 50 $TCID_{50}$ using the 1V-vectors or MOI 100 $TCID_{50}$ using the constructs containing $TetO_2$ regulated gRNAs. Plates were harvested at day zero and day two post infection. Readout was performed by determining total infectious units of each harvested sample.

Dual luciferase luminescence reporter assays

Before proceeding to recombinant virus-based inhibition experiments, the platform's knockdown ability was evaluated in luminescence reporter assays based on the Dual-Glo® system (Promega, Wisconsin, USA). The reporter plasmid psiCHECK-2 was restricted by XhoI within in the 3' UTR of Renilla luciferase and fused to large stretches of adenoviral *pol* and *pTP* coding sequences via the NEBuilder method, according to the protocol (New England Biolabs, Frankfurt am Main, Germany). Inserts were amplified from HAdV-5 with primers psiCHECK_POL_fwd 5'- cagtaattctaggcgatcgcCGCGGTCGTCAGGTCAAC-3'; psiCHECK_POL_rev 5'- gtcgcggacgGTTTCCATGAGCCGGTGTC-3'; psiCHECK_PTP_fwd 5'- tcatggaaacCGTCCGCGACTTTCCGCG-3'; psiCHECK_PTP_rev 5'- gatattttattgcggccagcCTAAAAGCGGTGACGCGGG-3'.

To check for knockdown efficiency 2 e + 04 HEK293 cells were seeded per well into 96-well plates and reverse transfected with CasRx and gRNA expressing plasmids, 200 ng each, together with 100 ng reporter plasmid, according to the Lipofectamine 2000 protocol (Thermo Fisher Scientific, Vienna, Austria). Readout of Firefly and *Renilla* luciferase activity followed after 48h according to the Promega protocol on a SpectraMax i3x plate reader (Molecular Devices, San José, USA).

Determination of pTP and pol mRNA levels

8e + 04 A549 cells, diluted in 500µL medium, were seeded per well in 24-well-plates and directly transduced with CasRx and gRNA expressing recombinant virus at an MOI of 100 $TCID_{50}$ each. HAdV-5 was added at an MOI of 0,01 $TCID_{50}$ 48 hours later. At 2 days post wild-type infection, total RNA was harvested using the RNeasy Kit according to the protocol (QIAGEN, Hilden, Germany). On column DNase digestion was included and the RNA was eluted in 35µL H_2O. cDNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Vienna, Austria). Subsequent quantitative real time PCR analysis was performed on a Quant Studio 7 system (Thermo Fisher Scientific, Vienna, Austria) using 1µL cDNA as template. The following primer/probe sets were used: pol-mRNA Pol-cDNA-f1 5'- ATGGCCTTGGCTCAAGCTC -3'; Pol-cDNA-r1 5'- GCGTAGGTTGCTGGCGAAC -3'; Pol-cDNA-p1 5'- CGCCTCTGCGTGAAGACGACGG -3' and Ptp-cDNA-f1 5'- GCCATGGCCTTGAGCGTC -3'; Ptp-cDNA-r1 5'- GGACGCGGTTCCAGATGTT -3'; Ptp-cDNA-p1 5'- AAAGTGCTCCATGGTCGGGACGCT -3' for ptp-mRNA as well as GAPDH-cDNA-f1 5'- TGCACCACCAACTGCTTAGC -3'; GAPDH-cDNA-r1 5'-

GGCATGGACTGTGGTCATGAG -3'; GAPDH-cDNA-p1 5'- CCTGGCCAAGGTCATCCATGACAACTT -3';GAPDH served as reference. The applied Blue Probe 2x Master mix was obtained from Biozym (Vienna, Austria). All PCRs were performed in duplicates and fold changes calculated using the ΔΔCT method.

Statistical analysis

Statistical significance was determined by one-way ANOVA, corrected with the Dunnett's post hoc test, in case of multiple comparisons, or by the student's t-test when comparing two values. A p value of <0,05 was considered statistically significant. The level of significance is indicated for each experiment. Data was analysed and graphs drawn using the Prism Software version 8.00 (GraphPad).

Results

Choice of target sites

The starting point was to select wild-type virus target genes that, when inhibited, are expected to have the biggest possible impact on viral replication, thus providing the prerequisite for a successful antiviral system. Simultaneously, risk of off-target effects and viral escape needed to be low. We chose *pTP*, coding for the pre-Terminal Protein and *pol*, the viral DNA polymerase. Both genes are expressed early in the replication cycle and essential parts of the DNA replication machinery (Leppard, 2008). The decision was supported by data showing successful inhibition of viral replication by RNAi targeting these genes (Ibrisimovic et al., 2013; Kneidinger et al., 2012). Target sites all lie within the coding regions of the respective genes. Two gRNAs per gene were designed. MRNAs of both genes are processed from the same primary transcript – E2B – (Donovan-Banfield et al., 2020; A. M. Price et al., 2022), and therefore share sequence homologies via their intronic and untranslated regions, expected to be targeted in addition to the coding sequence in mature mRNAs, as CRISPR/Cas is active within the nucleus. Figure 2 (Ma et al., 2016; Wolter & Puchta, 2018).

Antiviral defense: Applying the inherent function of CRISPR/CasRx

CasRx, the Cas13d of *Ruminococcus flavefaciens* XPD3002, is an RNA dependent RNA targeting multifunctional enzyme, belonging to class II type VI of CRISPR/Cas effector proteins. First described by Konermann et al., the CasRx coding sequence was originally found downstream of an CRISPR array (Konermann et al., 2018). These arrays are a collection of different sequences, having their origin in foreign genetic elements, interspaced by hairpins, the direct repeats. CasRx subsequently processes these arrays, producing gRNAs that when taken up by the CasRx enzyme, guide the way to an invader's RNA for cleaving Figure 3 A (C. Zhang et al., 2018). In research and proposed therapies this pathway can be exploited in two ways. Either by directly providing a processed version of an gRNA or by providing an array of multiple unprocessed ones. CasRx processes an array by cutting away 6 bases of the direct repeat 5' and 8 bases of the spacer 3', so there are differences in sequence design to be accounted for. In the designed array, a 30nt spacer is flanked by two 36nt long direct repeats, a processed gRNA on the other hand is made up by a 22nt spacer and a 30nt repeat 5' Figure 3 B (Konermann et al., 2018). In contrast to most other CRISPR/Cas systems, a PAM or PFS motif is not required when choosing target sites. Thus, a high level of flexibility is provided. Another advantage is the system's efficiency and specificity. Konermann et al. reported knockdown rates of 96% compared to 65% using RNAi *in vitro.* Off-target effects were dramatically reduced (Konermann et al., 2018; Yan et al., 2018; Y. Zhang et al., 2021).

Each gRNA sequence was checked by two independent online tools, GGGenome and TagScan, to prevent undesired shared homologies with the human transcriptome and possible off-target effects. No

hits with less than two mismatches were reported via TagScan. When using GGGenome, Pol-gRNA1 showed homologies to the human ACAT2 mRNA, although with three mismatches and one of them being in the seed region, nucleotide 15-21, reported by Wessels et al. to need perfect base complementarity (Wessels et al., 2020). All targeting guides have 100% sequence complementarity to adenovirus subtypes 1,2, 5 and 6 (U.S.NationalLibraryOfMedicine). The non-targeting control showed a desirable homology profile as well, with no reported candidates within the human transcriptome. A BLAST search at NCBI against the sequence collection *Adenoviriade* also revealed no significant hits making it a suitable negative control Figure 3 C.

Proof of principle: Testing prototype plasmids

We created prototype plasmids, one expressing CasRx and five others expressing individual gRNAs. Besides coding sequence, the CasRx component consists of several additional supporting modules. Two nuclear localisation signals, added up- and downstream of the protein, enhance transport into the nucleus and thus targeting activity. EGFP, split up from the enzyme by the ribosome skipping site T2A to prevent interference on protein level, serves as transduction control (Toutounchian & McCarty, 2017). CasRx is expressed under a tet-regulated CMV promoter, thus preventing unrestrained self-targeting during amplification in T-Rex-293 cells, stably producing the tetracycline repressor protein. The gRNA component is made up of a human U6 promoter, followed by the processed version of the direct repeat hairpin, the spacer and a p(T) terminator.

CasRx- and corresponding gRNA plasmids were tested for their knockdown efficiency using a dual luciferase reporter assay. The psiCHECK-2 reporter plasmid, part of the Dual-Glo assay available at Promega, produces two luciferase proteins - Firefly and *Renilla*. The coding sequence of *pol* and *pTP* was cloned downstream of *Renilla* and thus added to its mRNA during transcription. If targeted by CRISPR/CasRx, *Renilla* luminescence is lost, measured by a reduction of relative light units (RLU), which directly mirrors knockdown efficiency (Figure 4 A) (Promega; Sherf & Navarro, 1996). CasRx-, gRNA- and psiCHECK-2 plasmid DNA was co-transfected into HEK293 cells and readout 48h later. Results, normalized to non-targeting control, show highly significant knockdown across gRNAs. The highest inhibition rate is reached by Pol-gRNA2. Knockdown levels are between 60% and up to more than 80% for Pol-gRNA2 (Figure 4 B). The results demonstrate successful and significant mRNA knockdown of adenoviral sequences, in the range of or superior to previously reported rates using RNAi (Ibrisimovic et al., 2013), and thus lay the foundation for the next steps.

The optimized platform: All-in-one therapeutic viruses

The expression cassettes of CasRx and gRNA plasmids were combined. The resulting CasRx/gRNA expression cassettes were moved into the recombinant adenoviral vector. Regulating the expression of CasRx during virus amplification is exceptionally important as all constructs, except non-targeting

control, are self-targeting due to the presence of *pTP* and *pol*. While self-targeting is an issue, using a delivery vector of the wild-type's species also has advantages, as noted in the introduction, such as mobilization of the recombinant particle Figure S2. The gRNA without CasRx cannot mediate inhibition. Therefore, we initially only put CasRx under control of a tet-repressor. However, amplification of the indicated best performing Pol-gRNA2 containing vector was still heavily compromised, which probably was due to incomplete repression of the enzyme's expression.

Regardless of this, however, it was possible to generate recombinant virus of the other vector candidates and to test their performance in inhibition assays against wild-type virus. We designed two assay approaches, prophylactic and therapeutic. In the prophylactic approach, recombinant virus was applied prior to wild-type, transduced 48 h later. The readout was done at zero days, giving a baseline measurement of wild-type transduced, and at two, four and six days to follow replication over time. In the therapeutic approach, wild-type was added first and recombinant six hours later. Samples were harvested and evaluated two days later.

All targeting gRNAs showed highly significant inhibitory effect compared to the non-targeting control. For the prophylactic approach, inhibition rates of up to 1 order of magnitude were obtained at day 2 (Figure 5 A). Over the course of six days, we report inhibition rates in the range of 1 to two orders of magnitude (Figure 5 B). Evaluation of the therapeutic approach also showed highly significant repression rates across gRNA candidates. However, the were slightly reduced compared to the prophylactic approach. This is expected as the therapeutic approach is advantageous for wild-type virus infection (Figure 5 C).

Evaluation of the best performer in two vector system

Pol-gRNA2 had turned out to be the best performer in dual luciferase reporter assays but recombinant al-in-one CasRx/pol-gRNA2 vector could not be amplified in T-Rex 293 cells. However, before addressing this problem, recombinant virus was generated from the already made two component-based plasmids, applied in initial conducted proof of principle experiments. Not being susceptible to the self-targeting problematic, as gRNA and CasRx are being split onto two individual vectors, no setbacks were encountered when generating Pol-gRNA2 and other recombinant viruses.

Compared to non-targeting control, inhibition rates were around 1 order of magnitude. 2V-Pol-gRNA2 showed more than 1 order of magnitude repression of wild-type virus total viral progeny (Figure 6 A). At day 6 post infection, 2V-Pol-gRNA2 decreased HAdV-5 titers by around 2 orders of magnitude (Figure 6 B).

The ability of 2V-Pol-gRNA2 to significantly inhibit HAdV-5 replication was additionally reflected in a decrease of mRNA. We performed inhibition assays following the prophylactic approach and read out levels of *pTP* and p*ol* mRNA at 2 days post infection. We report highly significant reduction down to

around 10% residual mRNA levels, compared to non-targeting control in case of *pTP* mRNA. Interestingly this is also true for *pol* targeting constructs, 2V-Pol-gRNA2 showing a reduction down to less than 10% (Figure 7 A). Relative *pol* mRNA levels are reduced to 30% for 2V-Pol-gRNA1 and to less than 20% for 2V-Pol-gRNA2 (Figure 7 B).

The best performer in an all-in-one vector system

With Pol-gRNA2 performing best we intended to improve our all-in-one vector platform. To this end we modified the U6 promoter of Pol-gRNA2 and included two $TetO_2$ operators, to prevent expression during vector amplification in T-Rex 293 cells and to reduce the self-targeting possibly further. As it may be that the wild-type U6 promoter has a higher activity than the modified one (Henriksen et al., 2007) we also adjusted the non-targeting control vector. Indeed, this modification allowed the production of high levels of the Pol-Tet-gRNA2 vector. Investigating the self-targeting ability by comparing increase in vector infectious units of non-targeting NT-Tet-gRNA control and Pol-Tet-gRNA2, we observed a highly significant 2 orders of magnitude difference at two days post transduction. The control was amplified by 10.000 fold, while the targeting construct was only amplified by 100 fold (Figure S3). In the following virus inhibition assays, under prophylactic conditions, we saw an inhibition of about 1,5 orders of magnitude by the vector (Figure 8 A). In a time-course of over six days experiment we saw a stable trend of 2 orders of magnitude inhibition at all days (Figure 8 B). These results were repeated under therapeutic conditions, showing a highly significant inhibition of more than 1 order of magnitude (Figure 8 C). Especially striking is it when looking at the challenging therapeutic experiment design where we see an increase in inhibition potential as well. The data confirms the previous assumptions of CasRx-Pol-Tet-gRNA2 being the best performer and therefore the best candidate for future projects.

Discussion

Human pathogenic viruses remain a major challenge to the community, even more so in immunocompromised sub populations, highlighting the constant need for new and more efficient ways for treating viral infections (Englund et al., 2011). This medical need was brought to public attention in 2019 with the rising Sars-CoV-2 pandemic. Virology, previously an underrepresented branch with less than 2% research share, COVID related topics alone increased to about 10-20% of all biomedical research. Viral infections and diseases in general, together with public health and healthcare moved back into the spotlight as key social issues that need to be addressed (Harper et al., 2020).

In this study we employ CRISPR/CasRx to inhibit adenoviral replication. Adenovirus is a human pathogenic DNA virus causing life threatening diseases in high-risk populations (Lynch et al., 2011). DNA and RNA targeting CRISPR/Cas systems as well as RNAi have been successfully applied as means of antiviral treatments before (Liu et al., 2015; C. Wooddell et al., 2017; L. Yin et al., 2020). CasRx, a Cas13 type RNA targeting effector described by Konerman et al., was shown to be a highly efficient and specific, significantly surpassing characteristics of RNAi (Konermann et al., 2018).

The project's most important initial choices included which viral genes to target and how to deliver the proposed therapeutic platform to cells. We expected the greatest chance of success to lie in the selection of the essential and early expressed genes *pTP* and *pol*. We adapted target sequences, previously described in former in-lab projects (Ibrisimovic et al., 2013; Kneidinger et al., 2012) for use in our CasRx expression system. All were chosen as such to target multiple types of adenoviruses. Prior to the integration into our proposed antiviral platform, we checked for undesired sequence homologies which target sequences of the human genome, thus adding another layer of security against off-target effects in an already highly specific system. In case of the non-targeting control, we additionally sought to exclude any homologies to the viral genome. Target site locations all resided within the coding regions of the viral genes. For delivery, we employed established first-generation adenoviral vectors. However, it is clear that in future steps, going *in vivo* or at some point into the clinic, it is advised to switch to advanced generation vectors with reduced immunogenicity such as third generation vectors, also known as helper dependent or gutless vectors, or to AAV-based vectors (Chang, 2021; D. Wang et al., 2020) to target wild-type HAdV-5. Still, the decision to use adenovirus vectors to target wild-type HAdV-5 was made consciously in expectation of replication of recombinant vector upon encountering wild-type and thus an increase of CRISPR/Cas effector molecule in HAdV-5 infected cells.

The overall workflow included three types of constructs: i) two-vector based proof of principle platform, ii) an all-in-one vector system and iii) an optimized all-in-one vector system. The two-vector platform was used in dual luciferase reporter assays to answer the question whether CasRx in general is suitable for successfully targeting adenoviral mRNA sequences. **Figure 4** shows highly significant reductions in RLU across samples of up to more than 80% indicating that the system was indeed successful. However,

these experiments were conducted with non-replicating target sequences at low copy numbers and not with replicating virus. Thus, in the next step replicating virus had to be used.

To this end we switched to recombinant adenovirus particles, capable of delivering CasRx protein and gRNA within one vector. This was supposed to increase the functional dose and reduce mosaicism (i. e. cells carrying only CasRx or gRNA, respectively). Generation of recombinant virus was successful for all constructs except Pol-gRNA2, the best performer in previous reporter assays, presumably due to residual expression leading to self-targeting in packaging cells. The rest of the constructs could be tested in viral inhibition assays. We performed two types of assays - prophylactic and therapeutic, with the latter representing the more realistic scenario of treating an infection. All gRNAs reduced viral titer by up to one order of magnitude. This was true at two days post wild-type infection and also over the time course of six days. A similar picture was obtained when applying the therapeutic treatment scheme. This indicated that enough CRISPR/Cas effector molecule is generated within short time to allow efficient targeting of HAdV-5 mRNAs, even when the delivery of the respective vectors happens shortly after infection with wild-type HAdV-5 (Figure 5).

To be able to also evaluate Pol-gRNA2 we had to go one step back and employ the two-vector system in virus inhibition experiments. As self-targeting during vector amplification is no issue in this case (CasRx and gRNA expression cassettes located on separate adenoviral vectors), amplification of the Pol-gRNA2 vectors was feasible. The subsequent prophylactic inhibition assays revealed that Pol-gRNA2 was the best performer among the gRNAs tested (Figure 6).

Furthermore, *pTP* mRNA levels were highly significantly reduced by all four gRNAs to levels of about 10% remaining, and slightly below 8% for Pol-gRNA2. *Pol* mRNA levels were reduced to around 30% for Pol-gRNA1 and to less than 20% for Pol-gRNA2 (Figure 7). The cross-reactive activity (Pol-gRNAs reducing *ptp* mRNA levels), shown in Figure 7 A we think may be due to targeting of intronic or untranslated regions within the *ptp* pre-mRNA. We expect the same for pTP-gRNAs. See Figure 2 for more information. Reduction of total viral progeny might be another contributing factor.

In general, Pol-gRNA2 emerged as the most effective gRNA also in these assays. Consequently, the all-in-one vector system had to be modified to allow the expression of Pol-gRNA2 in combination with CasRx. To this end we added an additional $TetO_2$ operator upstream of the gRNA-encoding sequences to also reduce gRNA expression largely during vector amplification and to thus reduce self-targeting of the vector as much as possible. In subsequent assays we observed highly significant inhibition by Pol-gRNA2 of 1,5 orders of magnitude at day two post wild-type virus infection and up to more than 2 orders of magnitude at certain later time points. This translated into a total reduction of infectious viral particles by around 98% compared to the non-targeting control gRNA (Figure 8). We also saw further improvement compared to the previous results obtained with the all-in-one vector when applying the therapeutic treatment scheme (compare Figure 8C and Figure 5C).

The observed reduction rates were similar to that obtained with *pTP* and *pol* targeting siRNAs and amiRNAs published RNAi based results (Ibrisimovic et al., 2013; Kneidinger et al., 2012). While direct side-by-side comparisons have not been made, our results suggested that CasRx may be more effective than RNAi based approaches to inhibit adenovirus replication *in vitro*. Since RNAi is effective in inhibiting adenovirus replication *in vivo* (Schaar et al., 2017) CasRx may have the same potential. However, because not only the delivery of the gRNA-encoding sequences but also that of the CasRx effector protein is needed in this case, a direct comparison of the 2 approaches in an *in vivo* model in the future could be challenging.

Conclusion

In conclusion, CasRx lived up to expectations and can be successfully and efficiently be applied as means of targeting viral replication, highlighted by up to more than 2 orders of magnitude repression of HAdV-5 replication. This work contributes to current characterisation and knowledge of CRISPR/Cas systems and their possible application as therapy against diseases. CasRx may pose a promising candidate in future therapeutics addressing the rising medical need of answers to viral infections and epidemics.

Supplemental Information

Supplemental Information includes three figures: S1 - Cell viability and transduction efficiency across samples; S2 - Mobilization of recombinant adenovirus in the presence of wild-type HAdV-5; S3 – Assessment of vector self-targeting by Pol-gRNA2 as possible leading cause for amplification failure; and can be found with this article online at (to be announced)

Author Contributions

F.R., Z.D., A.W. and R.K. were responsible for the conception of the work and the design of the experiments. F.R. and Z.D. performed the experiments. F.R., Z.D. and R.K. analysed the data and wrote the paper. A.W. revised the work.

Conflicts of Interest

The authors declare no conflicts of interest

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Figure 1

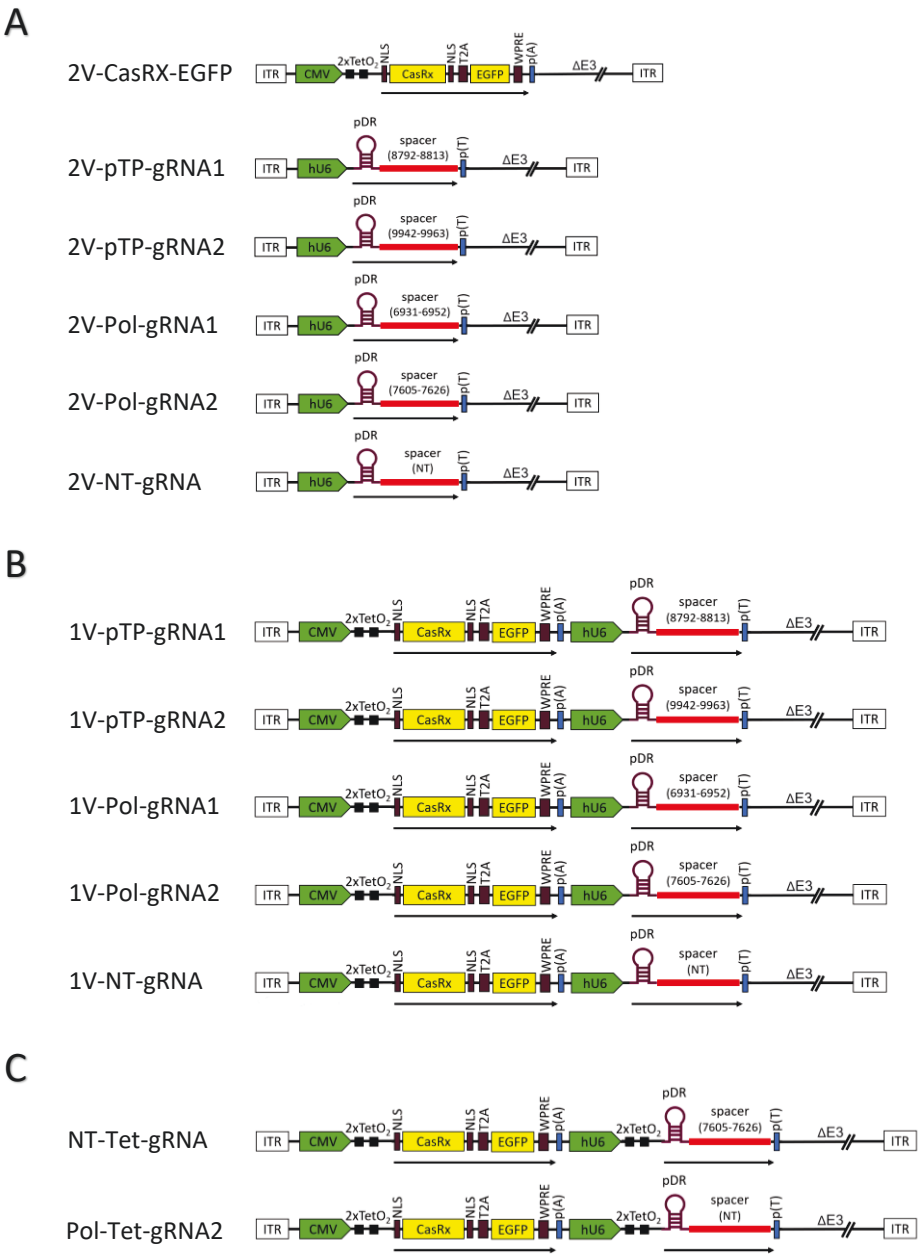


Figure 1 Design of expression cassettes. A: The initial proof of principle system consisted of two vectors. One expressing the effector CasRx under control of a tetracycline repressor regulatable CMV promoter downstream linked to EGFP with a T2A ribosome skipping site. EGFP served as control to determine transfection- and in later stages, transduction efficiency. A WPRE site served to enhance expression and nuclear localization signals to localize CasRx to the nucleus. The second vector type consisted of a set of five different gRNA cassettes. Two gRNAs each target adenoviral *ptp* and *pol* mRNA. A non-targeting gRNA served as control. The gRNAs were expressed under human U6 promoter, using processed versions of their direct repeat- and spacer regions. **B** shows the all-in-one expression system with CasRx and gRNA both lying within the same cassette and vector. The overall composition remained the same. As generating recombinant virus from 1V-Pol-gRNA2 was not possible, we modified the cassette as depicted in **C**. In order to support virus amplification by further reducing expected residual self targeting, we added tet operators to gRNA expression cassettes.
Each construct, making use of an entry- and destination plasmid based ΔE1/E3 adenoviral expression system, served as template for recombinant virus production in packaging cells.

Figure 2

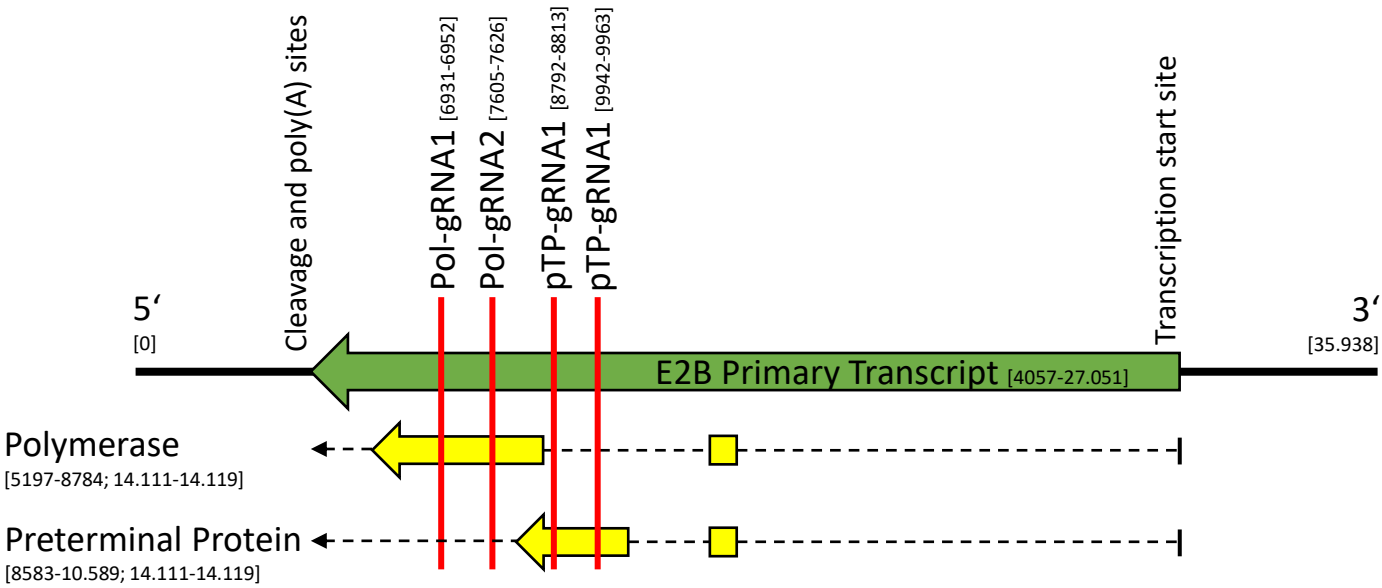
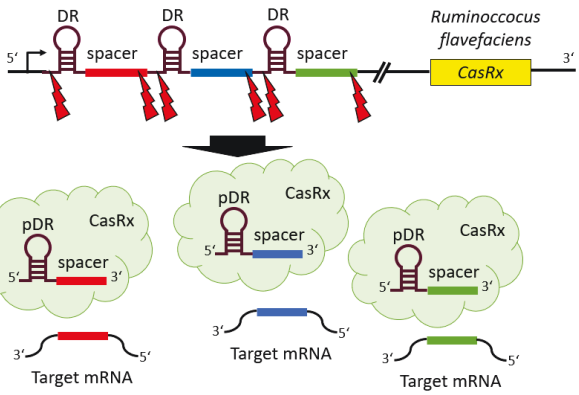


Figure 2 Location of gRNA target sites. Depicted in this picture is the adenoviral genome (NCBI reference genome AC_000008.1) with E2B primary transcript region, transcribed in 3' to 5' orientation, highlighted in **green.** *Pol* and *pTP* mRNAs, originating from E2B, with open reading frames in **yellow**, are indicated below. Two gRNAs were designed for each gene. The locations of gRNA target sites are indicated in **red.** Each gRNA targets the coding region of its intended gene and additional, intronic or untranslated regions (dotted lines) of the respective other gene's pre-mRNA. Information about Human Adenovirus 5 sequence locations drawn from Price et al., 2022.

Figure 3

A



B

processed Direct Repeat [30] variable spacer [22]

C

Target	Name	Sequence	Location	Specificity	TagScan up to 2-mm	GGGenome up to 3-mm
pTP(CDS) E2B PT	pTP-gRNA1	5′-AGAGTTCGACAGAATCAATTTC-3′	8792-8813	1, 2, 5, 6	0	0
pTP(CDS) E2B PT	pTP-gRNA2	5′-GTCTTGCATGAGCCTTTCTACC-3′	9942-9963	1, 2, 5, 6	0	0
Pol(CDS) E2B PT	Pol-gRNA1	5′-GGTTGGACGCTGGAAGACGTTG-3′	6931-6952	1, 2, 5, 6	0	1 (3-mm)
Pol(CDS) E2B PT	Pol-gRNA2	5′-TAAGTTCCTCGTAGGTGAGCTC-3′	7605-7626	1, 2, 5, 6	0	0
-	NT-gRNA	5′-TCACCAGAAGCGTACCATACTC-3′	-	-	0	0

Figure 3 Design of gRNAs and their spacer sequences. A: Within the *Ruminoccocus flavefaciens* genome a CRISPR array, consisting of a subset of different gRNAs, each with variable spacer regions, is followed downstream by the coding sequence of CasRx. The array, transcribed as one long RNA strand, is subsequently processed by the Cas enzyme. During this step, part of the direct repeat and spacer region is cleaved off, leaving behind individual gRNAs, each with a shortened direct repeat and spacer region **(B). Table C** summarizes the selected spacer sequences, their target and their target regions within the adenovirus genome. As *Adenoviridae* show sequence similarities across serotypes, spacers are specific not only for serotype 5 but also 1, 2 and 6. To reduce the risk of off-targets, each sequence was checked by TagScan and GGGenome for sequence similarities within the human genome, allowing for up to 2 or 3 mismatches (mm) respectively. Figure 3 A-B adapted from Konermann et al., 2018.

Figure 4

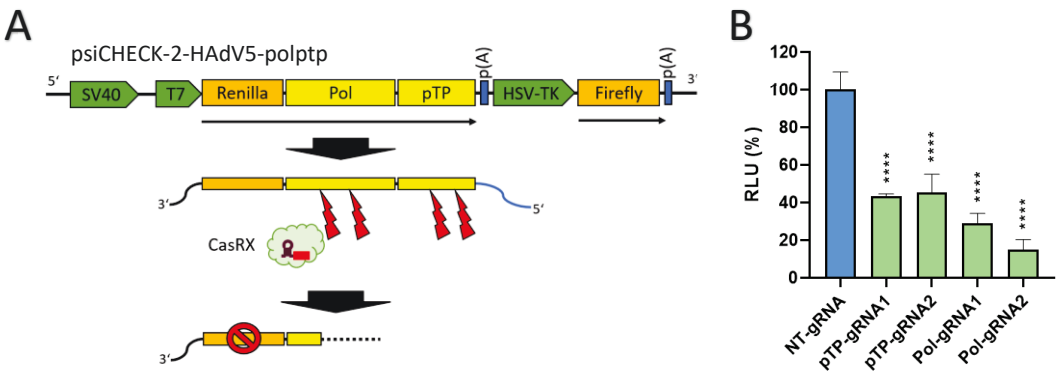


Figure 4 Dual luciferase assay of prototype plasmid transfections. Initial proof of principle for the proposed antiviral platform was generated by testing the two vector system (2V) in reporter assays, based on the Dual-Glo® system by Promega, Wisconsin, USA. **A:** Design of psiCHECK-2-HAdV5-polptp reporter plasmid and assay principle. The coding sequences of adenoviral *pol* and *pTP* are cloned into the transcriptional region of *Renilla* luciferase. Firefly luciferase serves as normalization control. If the *Renilla* transcript is targeted by CasRx effector molecules in one of four possible sites, depending on the respective gRNA, the stabilizing poly A region is cleaved off, the mRNA degraded and luciferase activity lost. Based on this reduction, the system's knockdown efficacy can be determined. **B:** HEK293 cells were transfected with CasRx and gRNA expressing plasmid DNA together with psiCHECK-2-HAdV5-polptp reporter plasmid. *Renilla* luciferase activity was read out 48h later, expressed as relative light units (RLU) in percent, with targeting gRNA values (**green**) normalized to non-targeting control (**blue**). Data, represents the means (n=3) ± SD of a representative experiment out of 3. ****$p < 0.0001$.

Figure 5

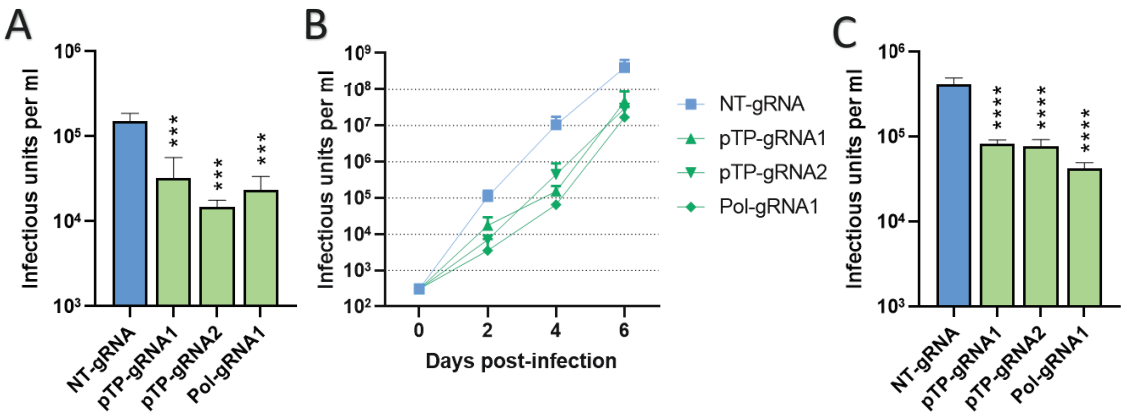


Figure 5 The all-in-one vector system (1V): Recombinant virus based inhibition of wild-type HAdV-5, prophylactic and therapeutic approaches. Following the prophylactic approach, A549 cells were transduced with recombinant 1V viruses at an MOI of 50 $TCID_{50}$ and 48h later infected with HAdV-5 at an MOI of 0,01 $TCID_{50}$. The number of infectious HAdV-5 units per ml was determined at days 0, 2, 4 and 6. **A** shows the readout at day 2 and **B** over the course of all time points. **C:** Following the therapeutic approach, A549 cells were infected with HAdV-5 at an MOI of 0,01 $TCID_{50}$ and six hours later transduced with recombinant 1V viruses at an MOI of 50 $TCID_{50}$. The number of infectious HAdV-5 units per ml was determined at day 2. Pol-gRNA2 virus could not be amplified with this vector design and thus is not present in the figure. Data represents the means (n=3) ± SD of a representative experiment out of 3, targeting gRNAs (**green**) and non-targeting gRNA (**blue**). ***p < 0.001, ****p < 0.0001.

Figure 6

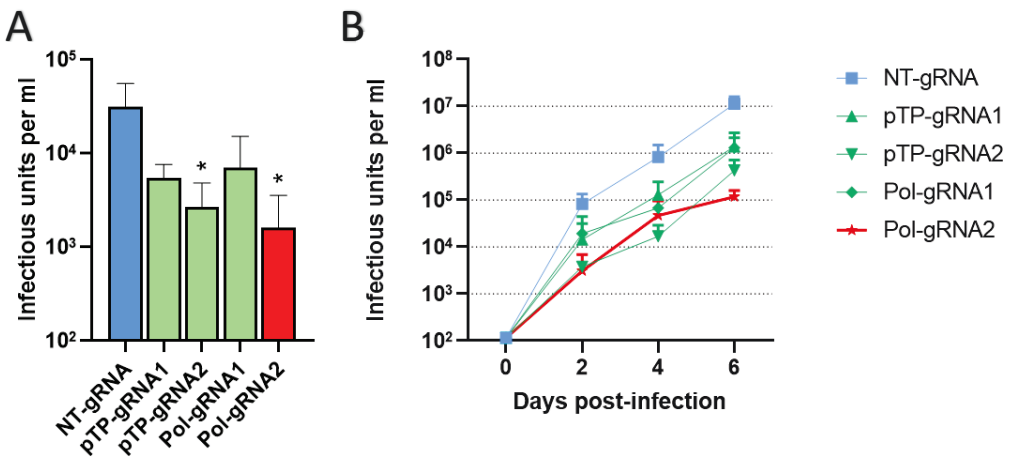


Figure 6 The two-vector system (2V): Recombinant virus based inhibition of wild-type HAdV-5 under prophylactic conditions to evaluate the best performer. Contrary to viral amplification of Pol-gRNA2 in all-in-one (1V) design, amplification in 2V design was feasible. A549 cells were transduced with recombinant 2V viruses at an MOI of 100 $TCID_{50}$ and 48h later infected with HAdV-5 at an MOI of 0,01 $TCID_{50}$. The number of infectious HAdV-5 units per ml was determined at days 0, 2, 4 and 6. **A** shows the readout at day 2 and **B** over the course of all time points with Pol-gRNA2 depicted in red. Data represents the means (n=3) ± SD of a representative experiment out of 3, targeting gRNAs (**green** and **red**) and non-targeting gRNA (**blue**). *p < 0.05.

Figure 7

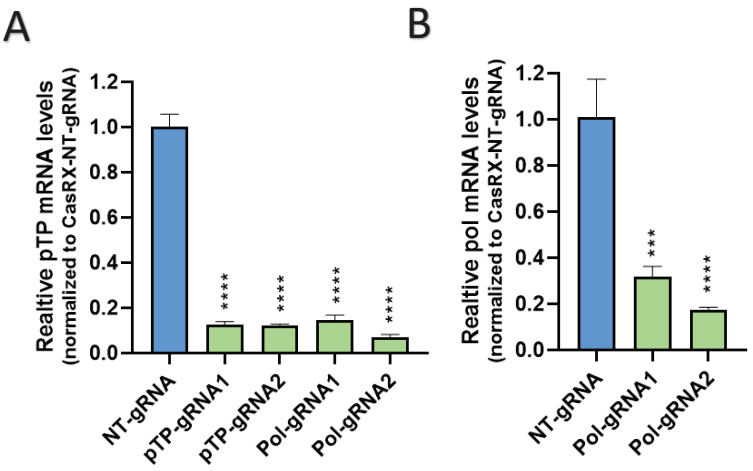


Figure 7 The two vector system (2V): Recombinant virus based inhibition of wild-type HAdV-5 mRNA under prophylactic conditions. A549 cells were transduced with recombinant 2V viruses at an MOI of 100 $TCID_{50}$ and 48h later infected with HAdV-5 at an MOI of 0,01 $TCID_{50}$. Total RNA was isolated at day 2 post HAdV-5 infection, cDNA obtained and relative *pol* and *pTP* mRNA levels evaluated by quantitative real time PCR. Reduction of mRNA levels by coding sequence (CDS) specific targeting of gRNAs shown for *pTP* mRNA in **A** and for *pol* mRNA in **B**. Cross-reactive targeting representatively shown for *pol* CDS specific gRNAs at *pTP* mRNA levels in **A**. Data represents the means (n=3) ± SD of a representative experiment out of 3, with targeting gRNAs (**green**) normalized to non-targeting gRNA (**blue**). ***p < 0.001, ****p < 0.0001.

Figure 8

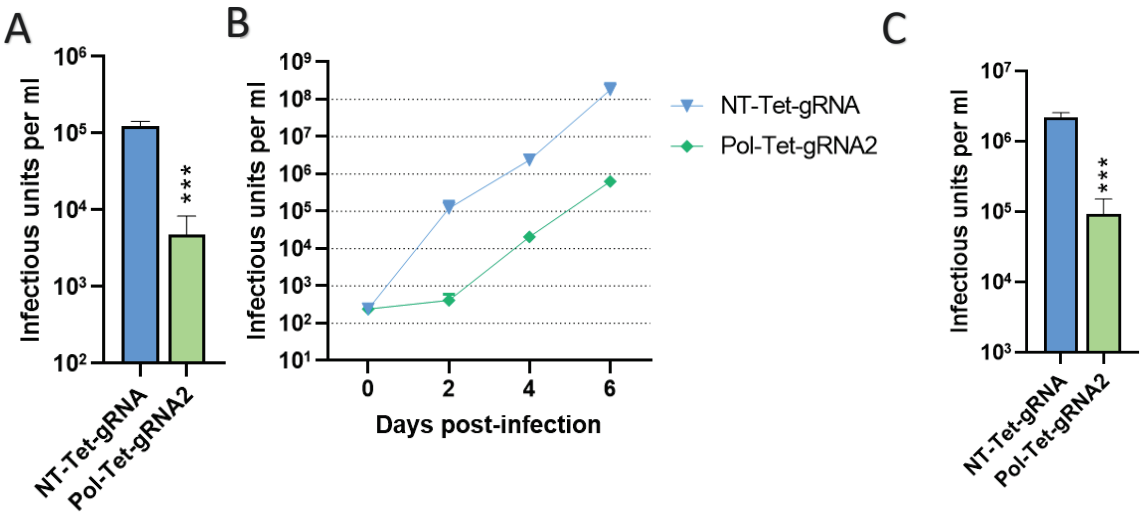


Figure 8 Pol-gRNA2 in all-in-one vector composition: Recombinant virus based inhibition of wild-type HAdV-5, prophylactic and therapeutic conditions. With tetracycline-repressor regulated gRNA expression, amplification of Pol-gRNA2 virus was feasible also in all-in-one vector design. For the prophylactic approach, A549 cells were transduced with recombinant viruses at an MOI of 100 $TCID_{50}$ and 48h later infected with HAdV-5 at an MOI of 0,01 $TCID_{50}$. The number of infectious HAdV-5 units per ml was determined at days 0, 2, 4 and 6. **A** shows the readout at day 2 and **B** over the course of all time points. **C:** For the therapeutic approach, A549 cells were infected with HAdV-5 at an MOI of 0,01 $TCID_{50}$ and six hours later transduced with recombinant viruses at an MOI of 100 $TCID_{50}$. The number of infectious HAdV-5 units per ml was determined at day 2. Data represents the means (n=3) ± SD of a representative experiment out of 3, targeting gRNAs (**green**) and non-targeting gRNA (**blue**). ***p < 0.001, ****p < 0.0001.

Figure S1

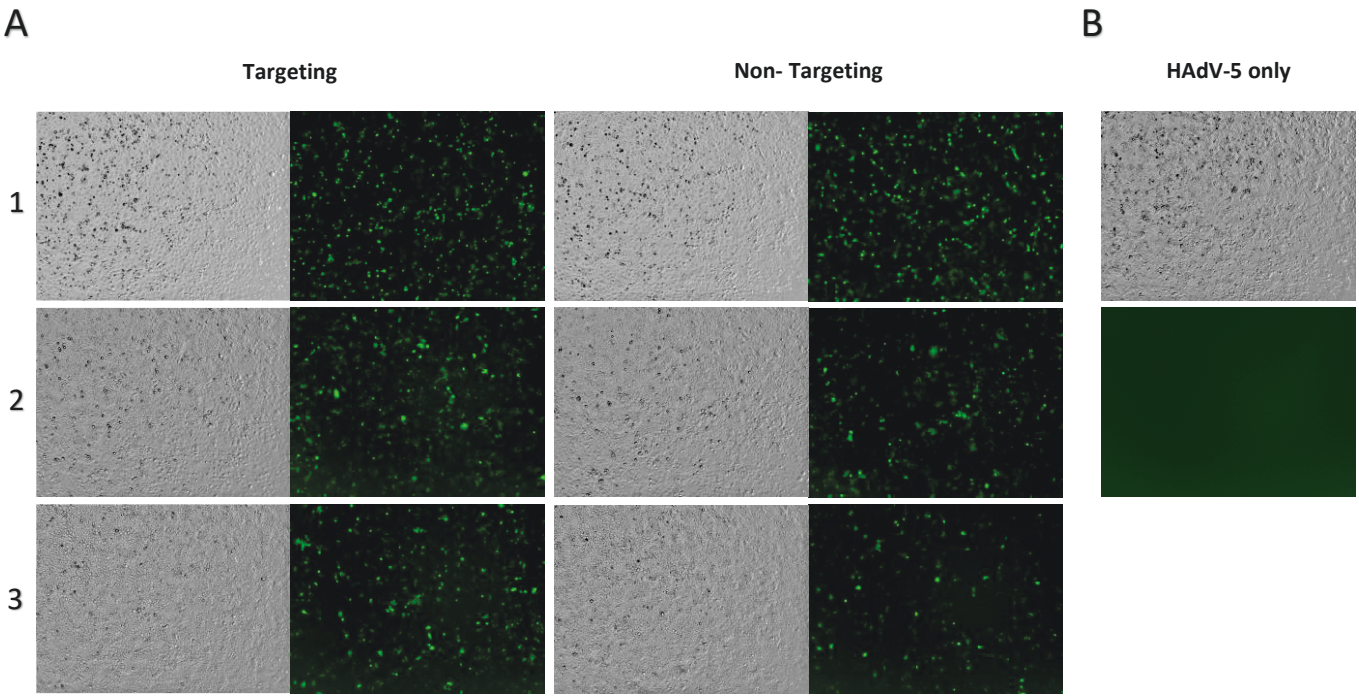


Figure S1 Cell viability and transduction efficiency across samples. Cell viability (bright-field microscopy pictures) and transduction efficiency (fluorescence microscopy pictures) of targeting gRNA samples (**A-left**), controls (non-targeting gRNA: **A-right**, wild-type HAdV-5 only: **B**) and assays (all-in-one vector system (1V) **A.1**, two-vector system (2V) **A.2,** and Tet-gRNA constructs **A.3)**. The figure shows cell conditions at two days post wild-type HAdV-5 infection of representative inhibition assays. Microscope pictures were taken on a SpectraMax i3x plate reader (Molecular Devices, San José, USA).

Figure S2

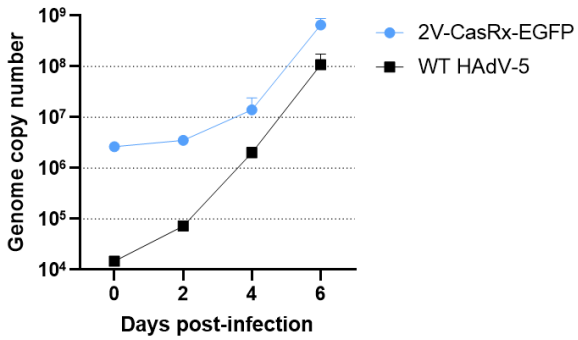


Figure S2 Mobilization of recombinant adenovirus in the presence of wild-type HAdV-5. A549 cells were transduced with 2V-CasRx-EGFP virus, as a representative of replication incompetent recombinant viruses, at an MOI of 100 $TCID_{50}$ and 48h later infected with HAdV-5 at an MOI of 0,01 $TCID_{50}$. DNA was isolated at day 0, 2, 4 and 6 post HAdV-5 infection and the total viral genome copy number evaluated by real time quantitative PCR using genotype specific primers. Data represents the means (n=3) ± SD of a representative experiment out of 3, with recombinant 2V-CasRx-EGFP vector in **blue** and wild-type HAdV-5 in **black**.

Figure S3

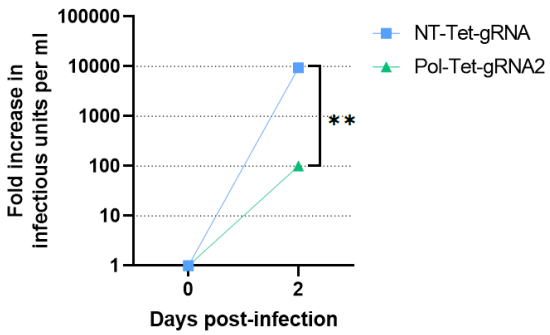


Figure S3 Assessment of vector self targeting by Pol-gRNA2 as the possible leading cause for amplification failure. HEK293 cells, permissive for recombinant vector replication and CasRx effector expression, were transduced with Pol-Tet-gRNA2 and NT-Tet-gRNA virus at an MOI of 0,1 $TCID_{50}$. The number of infectious units per ml (IFU/mL) was determined at days 0, 2 and normalized to NT-Tet-gRNA virus. Depicted in the figure are the fold increases in IFU/mL from day 0 to day 2. Data represents the means (n=3) ± SD of a representative experiment out of 3, targeting gRNA (**green**) and non-targeting gRNA (**blue**). **$p < 0.01$.

3.3 Combining CRISPR/CasRx and RNAi to inhibit adenovirus replication

3.3.1 Background and rationale

Having established an optimized CasRx based antiviral platform to inhibit adenoviral replication (see manuscript 3.2.), I investigated further options for improvement. Ibrisimovic et al., 2013, applying RNAi, reported successful inhibition of adenovirus replication by artificial micro RNAs *in vitro*. Inhibition rates were similar to those demonstrated by CasRx in this study. Therefore, I combined the two technologies to evaluate ways of multiplexing and their synergistic potential in treating adenovirus infection.

3.3.2 Materials and Methods

Cell culture

HEK293 (human embryonic kidney; ATCC CRL-1573) were cultured in Dulbecco's Modified Eagles Medium (DMEM) with high glucose and GlutaMAX and supplemented with 10% heat inactivated fetal bovine serum (Thermo Fisher Scientific, Vienna, Austria). They were grown in a humidified 5% CO_2 atmosphere at 37 °C.

Construction of CasRx and RNAi multiplexed plasmids

In order to evaluate different combination approaches, I designed 5 sets of constructs: 1) The amiRNA and a processed gRNA under a tetracycline-repressor regulated U6 promoter, 2) The amiRNA and a unprocessed gRNA under a tetracycline-repressor regulated U6 promoter, 3) Six amiRNAs together with CasRx under the same tetracycline-repressor regulated CMV promoter without stabilizing structures in between, and a U6-expressed gRNA, 4) Six amiRNAs together with CasRx under the same tetracycline-repressor regulated CMV promoter with WPRE in between, and a U6-expressed gRNA, 5) Six amiRNAs together with CasRx under the same tetracycline-repressor regulated CMV promoter with MALAT1 in between **(Figure 1-A)**. Basis for plasmid sequences were previously constructed vectors (see manuscript 3.2.), published data from Konermann et al., 2018 and amiRNA vectors from Ibrisimovic et al., 2013. All plasmids include targeting or non-targeting combinations of gRNA and

amiRNA (Figure 1-B). GRNA spacer sequences were taken from Pol-gRNA2 (targeting) and NT-gRNA (non-targeting) found in manuscript chapter 3.2. AmiRNA sequences are ptpmi5 (targeting) and pmiRE (non-targeting) from Ibrisimovic et al., 2013.

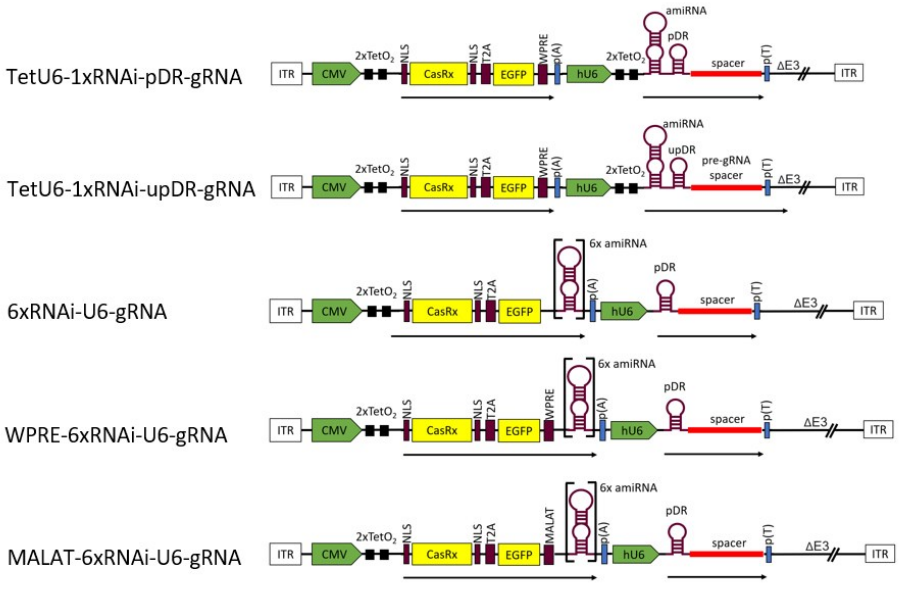
The RNAi-gRNA cassette of vector types 1 and 2 was ordered online (Biomatik, Delaware, USA), with flanking *Asc*I and *Xba*I restriction sites. The backbone (2V-CasRx-EGFP from manuscript 3.2.) and expression cassettes were restricted by *Asc*I and *Xba*I and ligated using the T4 Ligase according to standard protocol (New England Biolabs, Frankfurt am Main, Germany).

Type 3 was constructed by acquiring the backbone from 1V-Pol-gRNA2 (chapter 3.2) via the primers CRx-gRNA_Backbone_fwd 5'-cacatggaacaaatggcccaATCGATACCGTGCAGGATG-3' and CRx-gRNA_Backbone_rev 5'-agccttcagcaagcctccagATCGATAAGCTTGATATCGAATTC-3', excluding the WPRE sequence. The 6x-amiRNA cassette was acquired by restricting plasmids pcDNA6.2_6xptpmi5 and pcDNA6.2_6xpmiRE (Ibrisimovic et al., 2013) by *Bam*Hi and *Bgl*II. Backbone and amiRNA cassette were subsequently ligated via the NEBuilder approach (New England Biolabs, Frankfurt am Main, Germany).

Type 4 plasmids were generated by NEBuilder mediated insertion of the WPRE sequence, acquired from EF1A-CasRx-2A-EGFP-WPRE (Konermann et al., 2018) by the primers WPRE_insert_fwd 5'-caagtaagaattcgatatcaAATCAACCTCTGGATTACAAAATTTG-3' and WPRE_insert_rev 5'-agcaagcctccagatcgataACTTGTGATTGCTCCATG-3', into type 3 plasmids, restricted by *Hind*III.

Type 5 plasmids were generated equivalent to type 4, acquiring the Malat1 sequence by PCR from cGFP-mMALAT1_3' WT Sense (Wilusz et al., 2012), using primers Malat1_Insert_fwd 5'-caagtaagaattcgatatcaGATTCGTCAGTAGGGTTGTAAAGGTTTTTCTTTTC-3' and Malat1_Insert_rev 5'-agcaagcctccagatcgataCAGAAGCCCCTGCGCTGG-3'. At a later stage they were adapted with tetracycline-repressor regulated U6 promoters to prepare for recombinant virus amplification. This was achieved by Q5 site directed mutagenesis as well, using the oligos Change_TetU6_F 5'-atatctccctatcagtgatagagACCGAACCCCTACCAACT-3' and Change_TetU6_R 5'-ataatctctatcactgatagggagTTTCAAGTTACGGTAAGCATATG-3'.

A



B

Construct Candidate	RNAi[1]	CasRx[2]	Notes
TetU6-1xRNAi-pDR-gRNA_NT-NT	NT	NT	One amiRNA and processed gRNA under hU6_TetO$_2$ promoter.
TetU6-1xRNAi-pDR-gRNA_T-T	T	T	
TetU6-1xRNAi-upDR-gRNA_NT-NT	NT	NT	One amiRNA and unprocessed gRNA under hU6_TetO$_2$ promoter.
TetU6-1xRNAi-upDR-gRNA_T-T	T	T	
6xRNAi-U6-gRNA_NT-NT	NT	NT	Six amiRNAs 3' of EGFP, together with CasRx under CMV_TetO$_2$ promoter. CasRx gRNA under hU6. No stabilizing motif.
6xRNAi-U6-gRNA_T-T	T	T	
6xRNAi-U6-gRNA_NT-T	NT	T	
6xRNAi-U6-gRNA_T-NT	T	NT	
WPRE-6xRNAi-U6-gRNA_NT-NT	NT	NT	Six amiRNAs 3' of EGFP, together with CasRx under CMV_TetO$_2$ promoter. CasRx gRNA under hU6. WPRE stabilizing motif.
WPRE-6xRNAi-U6-gRNA_T-T	T	T	
WPRE-6xRNAi-U6-gRNA_NT-T	NT	T	
WPRE-6xRNAi-U6-gRNA_T-NT	T	NT	
Malat-6xRNAi-U6-gRNA_NT-NT	NT	NT	Six amiRNAs 3' of EGFP, together with CasRx under CMV_TetO$_2$ promoter. CasRx gRNA under hU6. MALAT1 stabilizing motif.
Malat-6xRNAi-U6-gRNA_T-T	T	T	
Malat-6xRNAi-U6-gRNA_NT-T	NT	T	
Malat-6xRNAi-U6-gRNA_T-NT	T	NT	

[1]RNAi targeting (T) or non-targeting (NT); [2]CasRx targeting (T) or non-targeting (NT)

Figure 1 Design of combinatorial expression cassettes. A: To evaluate different ways of combining CasRx and RNAi expression, five types of plasmids were designed. The first two types express an amiRNA fused to a processed or unprocessed version of the gRNA. After transcription the amiRNA and gRNA are divided either by DROSHA cleavage or CasRx processing, or a combination of both. The other three types express a six times amiRNA cassette fused to the CasRx transcript. When DROSHA cleaves off the amiRNAs, the CasRx containing mRNA is without stabilising poly A tail. Therefore the lower two types are supplemented with stabilizing structures (WPRE, MALAT1) 5' of the amiRNA cassette. The backbone of all constructs is formed by an entry- and destination plasmid based ΔE1/E3 adenoviral expression system for subsequent production of recombinant virus. All plasmid types include a combination of targeting and non-targeting amiRNAs and gRNAs, summarized in **B**.

Amplification and extraction of circular plasmid DNA

Up to 3µl of ligation reaction were added to 30µl of electro-competent NEB-10β (New England Biolabs, Frankfurt am Main, Germany) which were thawed on ice for 10min. The mixture was pulsed in electro poration cuvettes with a 0,1 cm gap using 2kV at a MicroPulse Electroporator (Bio-Rad, Hercules, CA,USA). 970µl of warm SOC medium was added and the cells incubated for 1 hour at 37°C. Subsequently, they were plated onto LB-Agar, supplemented with the corresponding selection antibiotic. 24h later, colonies were picked into 20µl of nuclease free water and colony PCR performed using the OneTaq® 2X Master Mix (New England Biolabs, Frankfurt am Main, Germany). Clones were evaluated by gel electrophoreses and the correct candidates incubated overnight in liquid broth, supplemented with antibiotic and harvested the next day. The DNA was extracted by the FavorPrep Plasmid Extraction Mini Kit (Favorgen, Vienna, Austria) or by the QIA Filter Plasmid Midi Kit (QIAGEN, Hilden, Germany) according to supplied protocols. The final plasmids were confirmed by restriction digest and sequencing at Microsynth, Balgach, Switzerland.

Dual luciferase signal reporter plasmids

To evaluate the different approaches of combining CasRx and RNAi within one expression vector, the plasmids were evaluated in dual luciferase signal reporter assays based on the Dual-Glo® system (Promega, Wisconsin, USA). Two types of reporter vectors, based on the supplied psiCHECK-2 vector, were used. The psiCHECK-2-HAdV5-polptp vector, having the coding sequence of adenoviral *pol* upstream of *pTP* (described in manuscript chapter 3.2.) and the newly constructed psiCHECK-2-HAdV5-ptppol, having *pTP* upstream of *pol*. Both coding sequences are cloned into the 3' untranslated region of the luciferase *Renilla.* If the sequences are targeted by either CasRx or RNAi, the mRNA is degraded and *Renilla* activity lost, directly mirroring knockdown efficiency of the proposed antiviral system.

The reporter plasmid psiCHECK-2-HAdV5-ptppol was constructed by the restriction of the psiCHECK-2 vector by *Xho*I and the subsequent ligation with the *pTP-pol* insert, obtained by PCR from HadV-5 using primers psiCHECK_POL_fwd 5'- cagtaattctaggcgatcgcCGTCCGCGACTTTCCGCG-3' psiCHECK_POL_rev 5'- gacgaccgcgCTAAAAGCGGTGACGCGGG-3' psiCHECK_PTP_fwd 5'- ccgcttttagCGCGGTCGTCAGGTCAAC-3'

psiCHECK_PTP_rev 5'- gatattttattgcggccagcGTTTCCATGAGCCGGTGTC-3', via an NEBuilder reaction.

To evaluate the mRNA knockdown efficiency, 2 e + 04 HEK293 cells were seeded per well into 96-well plates and transfected with 200ng of CasRx/amiRNA expressing plasmid DNA and 50ng of reporter

plasmid DNA, according to Lipofectamine 200 protocol (Thermo Fisher Scientific, Vienna, Austria). The readout was performed 48h later on a SpectraMax i3x plate reader (Molecular Devices, San José, USA).

Statistical analysis

The drawing of graphs and the statistical analysis was done using Prism Software version 8.00 (GraphPad, San Diego, USA). The level of significance, indicated in figures, was determined by one-way ANOVA, corrected with the Dunnett's post hoc test in multiple comparisons. The comparison of two values was done by the student's t-test.

3.3.3 Results

The dual luciferase reporter assay, including all constructed plasmids, with targeting and non-targeting combinations of amiRNA and gRNA, revealed no obvious differences when comparing between results using psiCHECK-2-HAdV5-ptppol or psiCHECK-2-HAdV5-polptp (compare Figure 2 A and B). Comparing plasmid types, TetU6-1xRNAi-pDR-gRNA reduced relative light units to around 25%, being more efficient than TetU6-1xRNAi-upDR-gRNA at around 35%. Plasmid types, expressing a six times amiRNA cassette together with CasRx, reduce relative light units to around 15% in case of constructs with stabilizing motifs and to 20-35% in case of plasmid 6xRNAi-U6-gRNA without. Trends of an additive effect may be present in certain cases, but no significant difference is observed between combinations of targeting and non-targeting amiRNAs and gRNAs.

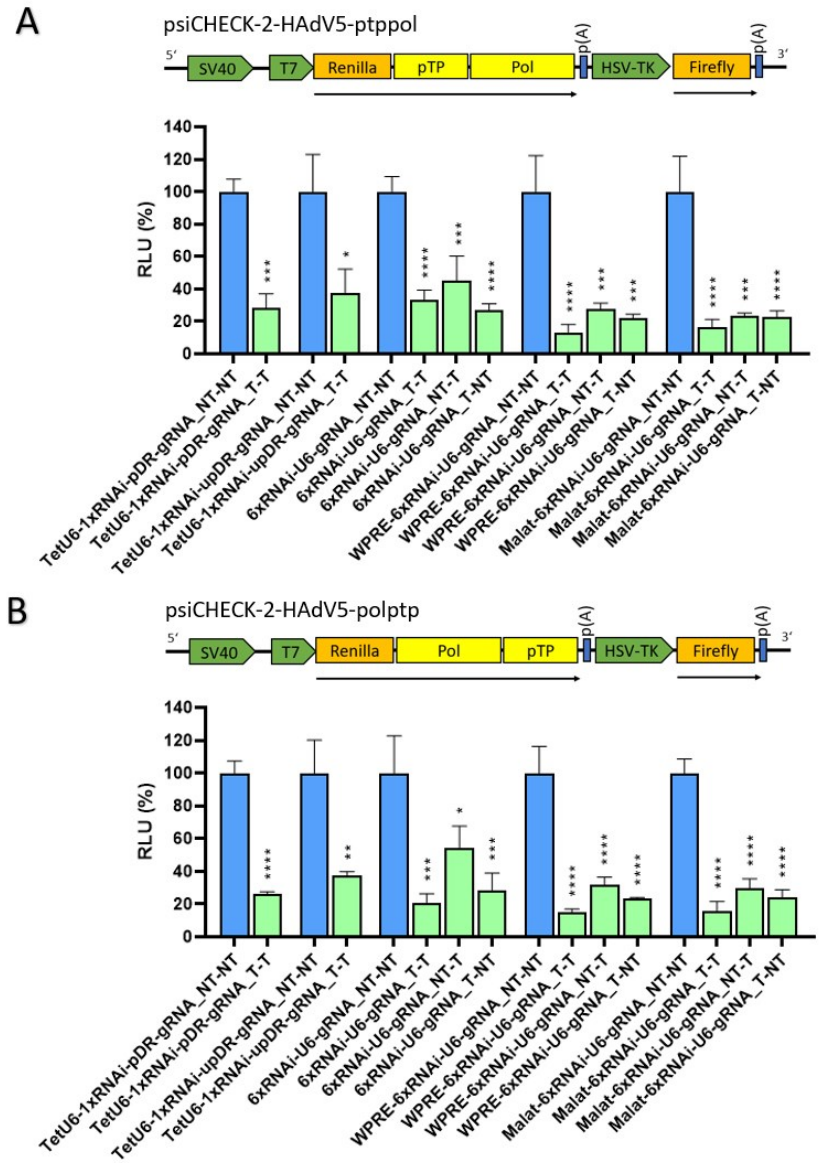


Figure 2 Dual luciferase signal reporter assay: Evaluation of combinatorial approaches HEK293 cells were transfected with CasRx-amiRNA expression plasmids and psiCHECK-2-HAdV5-ptppol **(A)** or psiCHECK-2-HAdV5-polptp (see manuscript 3.2.) reporter **(B)**. Both reporter vectors have the HAdV-5 *pTP* and *pol* coding sequence fused to the transcript of *Renilla* luciferase. If targeted by amiRNA or CasRx the mRNA is degraded, which can be measured as a reduction of *Renilla* activity (Promega, Wisconsin, USA). Values are expressed as relative light units (RLU) in percent, with targeting plasmids (**green**) normalized to non-targeting controls (**blue**). Data, represents the means (n=3) ± SD of a representative experiment out of 3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.3.4 Discussion

The CRISPR/Cas and RNAi technologies have been successfully applied numerous times as means of antiviral treatment in lab and clinical studies (see chapters 0, 1.4.4, and 1.5). We therefore hypothesized that the combination of both may lead to additive and synergistic effects. Ibrisimovic et al. 2013 showed such effects when targeting adenoviral replication by amiRNAs and cidofovir. Wang et al. investigated combinatorial targeting of Hepatitis B and Zhao et al. that of HIV by CRISPR/Cas9 and RNAi (J. Wang et al., 2017; C. Zhao et al., 2017). In our manuscript (chapter 3.4.) we show ways of Cas9-amiRNA multiplexing and report successful HAdV-5 inhibition using Cas9 in combination with cidofovir. To our knowledge CasRx, was not yet applied together with RNAi and the information on how to combine the two technologies most efficiently is missing.

Therefore, I constructed five different types of plasmids following two general approaches, with RNAi targeting HAdV-5 *pTP* and CasRx targeting HAdV-5 *pol* (**Figure 1**). The first two types express the amiRNA upstream of the gRNA, together under a tet-repressor regulated human U6 promoter. Expression of RNAi under its own promoter would have been also possible, however we chose the optimized multiplexed expression to limit the necessary capacity of a subsequent delivery vehicle i.e., recombinant adenoviral vector. The fused amiRNA and gRNA transcript is going to be cleaved by DROSHA within the nucleus producing the pre-miRNA and leaving the gRNA with overhangs (J. Wang et al., 2017). The gRNA may then be processed by CasRx, and the overhangs cleaved off (Konermann et al., 2018). However, it is difficult to predict what is happening in detail – if it makes a difference if DROSHA processing happens before that of CasRx or vice versa or whether the generated gRNA overhangs are a hindrance for CasRx processing and efficacy. In the type 2 plasmid (TetU6-1xRNAi-upDR-gRNA) unlike in type 1, I applied the unprocessed version of the gRNA, hypothesizing that if DROSHA-generated overhangs are indeed a hindrance to subsequent CasRx processing, the unprocessed type of gRNA, being closer to the natural occurring sequence, may support CasRx function.

In the other three plasmid types I inserted a six times amiRNA cassette, described by Ibrisimovic et al., 2013, into the CasRx expression cassette, downstream of EGFP. In this case DROSHA cleavage of amiRNAs is going to leave the CasRx transcript without stabilizing poly A (6xRNAi-U6-gRNA). In plasmids WPRE-6xRNAi-U6-gRNA and Malat-6xRNAi-U6-gRNA I addressed this expected issue by adding WPRE (Mahonen et al., 2007) or MALAT1 (Campa et al., 2019; Wilusz et al., 2012) upstream of the amiRNA cassette. Both are stabilizing structures that may supplement the missing poly A.

I tested all plasmid types, including combinations of targeting and non-targeting amiRNA and gRNA, in dual luciferase reporter assays to identify the best approach (Figure 2). I did this using two different reporter plasmids, both containing HAdV-5 *pTP* and *pol* coding sequences. However, the order of *pTP* and *pol* I switched between the two reporters to investigate the targeting process in more detail. CasRx targets mRNA within the nucleus. If CasRx-targeted mRNA would be subsequently exported, fragments theoretically could be targeted again by RNAi in the cytoplasm (see 1.3.2 and 1.4.1). The likelihood of that happing however is debatable as it would conflict with current understanding of the RNA export machinery (Carmody & Wente, 2009). Still, I applied these two reporter plasmids to investigate this question more closely. The RNAi effect may be more pronounced when it's specific coding sequence (*pTP)* is closer to *Renilla*. Unfortunately, no conclusions could be drawn on that matter as no significant differences were observed using one or the other reporter (compare Figure 2 A and B). Individual extraction of mRNA from the nucleus and the cytoplasm may give more insights in the future.

Looking at the knockdown rates of *pTP* and *pol* containing reporter mRNA, comparing plasmid type 1 (TetU6-1xRNAi-pDR-gRNA) and 2 (TetU6-1xRNAi-upDR-gRNA), there is a trend of the former design being the more efficient one. In case of the other vector types, the ones that supplement stabilizing structures (WPRE, MALAT1) are better performing then the plasmid type without.

Therefore, the best candidates are the processed gRNA approach (type 1 plasmid) when expressing amiRNA and gRNA together under one promoter and plasmid type 4 and 5 when expressing amiRNA together with CasRx. Furthermore, the type 1 expression cassette may also benefit from adding additional amiRNAs similar to type 4 and 5. Concerning an additive effect, there may be a slight trend, but final conclusions need to be drawn from the systems application in wild-type HAdV-5 inhibition assays. Due to time constraints however, I was not able to pursue this project further.

3.4 Manuscript: Inhibition of adenovirus replication by CRISPR/Cas9-mediated targeting of the viral E1A gene

Inhibition of adenovirus replication by CRISPR/Cas9-mediated targeting of the viral E1A gene

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Short title: Targeting of adenovirus by CRISPR/Cas9

ABSTRACT

DNA-targeting CRISPR/Cas systems are able to cleave dsDNA in mammalian cells. Accordingly, they have been employed to target the genomes of dsDNA viruses, mostly when present in cells in a non-replicative state with low copy numbers. However, the sheer amount of viral DNA produced within a very short time by certain lytically replicating viruses potentially brings the capacities of CRISPR/Cas systems to their limits. Accessibility of viral DNA replication sites, short time of accessibility of the DNA before encapsidation or its complexation with shielding proteins are further potential hurdles. Adenoviruses are fast-replicating dsDNA viruses, for which no approved antiviral therapy currently exists. We evaluated the potency of CRISPR/Cas9 in inhibiting the replication of human adenovirus 5 *in vitro* by targeting its master regulator *E1A* with a set of guide RNAs and observed a decrease in infectious virus particles by up to three orders of magnitude. Target DNA cleavage also negatively impacted the amount of viral DNA accumulated during the infection cycle. This outcome was mainly caused by specific deletions, inversions, and duplications occurring between target sites, which abolished most E1A functions in most cases. Additionally, we compared the two strategies for multiplex gRNA expression and obtained comparable results.

INTRODUCTION

After their discovery, DNA-recognizing CRISPR/Cas systems[1] were rapidly developed into tools for editing cellular DNA.[2-4] In the engineered versions, a single guide RNA (gRNA) pilots Cas9 to the target sequence residing adjacent to a so-called "protospacer adjacent motif" (PAM; 5'-NGG-3') followed by the cleavage of the dsDNA and the repair of the break via non-homologous end joining (NHEJ). As a result, small insertions or deletions (indels) occur at the cleavage site, which can result in frameshifts that render the affected protein inactive. In addition, the homology-directed repair (HDR) pathway is activated and can be exploited to insert DNA into target sites. CRISPR/Cas systems have also been evaluated for inactivation of the (pro)viral DNA of viruses such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), hepatitis B virus (HBV), herpes viruses, and human papillomavirus (HPV).[5,6] However, in most of these cases, the viral DNA was targeted while present in a non-replicative state, either incorporated into the host chromosome or present in a stable extrachromosomal form with low copy numbers. In these studies, the main goal was to eradicate viral genomes from infected cells, as they resist elimination by conventional drugs.

Targeting of actively replicating DNA viruses may potentially be complicated by several factors: (i) the viral DNA must be in a state that allows recognition by CRISPR/Cas9 (i.e., it must not be complexed with proteins that prevent the interaction), (ii) a spatial separation of the viral replication sites and the CRISPR/Cas9 effectors might make contact between them impossible, (iii) the time span during which the CRISPR/Cas9 components have access to the viral DNA before it is encapsidated again might be too short, and (iv) the inherent CRISPR/Cas9 potency might not be sufficient to allow these systems to cope with the sheer amount of DNA produced by certain viruses during lytic infection. To date, only a few DNA viruses have been investigated for targeting by CRISPR/Cas9 during lytic infection. This includes, vaccinia virus,[7] African swine fever virus,[8] polyomavirus JC[9,10] and a few examples of *de novo* infections of cells with viruses that are primarily investigated for being targeted during persistent infections such as herpes viruses and HBV, for example.[11-15]

Adenoviruses[16,17] contain dsDNA genomes, which render them suitable for targeting by CRISPR/Cas9. Human adenoviruses are associated with a variety of clinical symptoms, mostly

affecting the respiratory and intestinal tracts but also the eye. Infections are mostly self-limiting but can become serious and even life-threatening in immunocompromised patients.[18-21] Currently, no approved antiviral therapies for adenoviruses exist; hence, the treatment relies on repurposing of drugs for the treatment of other viral diseases[22] such as cidofovir (CDV) and derivatives, which, however, show limited efficacy, cause toxicity or are still under investigation.[23-26] Thus, alternative treatment options are needed and CRISPR/Cas9 is theoretically conceivable to be developed into a therapeutic to treat localized infections such as infections of the eye or even disseminated infections. In such therapeutic scenarios delivery may be based on viral or non-viral DNA or RNA.

In permissive cells, adenoviruses multiply productively and lyse their hosts. Fast-replicating human adenoviruses, such as human adenovirus 5 (HAdV-5), reach burst sizes of 1e+04 infectious units per cell or more. The central viral regulator of the infection cycle is the early region 1A (*E1A*) gene,[27-32] the first gene to be expressed after infection, which is needed to transactivate the expression of other viral genes.[33] In addition, E1A interacts with a large number of cellular targets[30] thereby manipulating their function with broad consequences for the cellular transcriptome, proteome, and interactome to create an environment that is beneficial for the virus.[30,31] Most E1A functions reside in four conserved regions (CR1–CR4).[34] Differential splicing of *E1A* RNA gives rise to several isoforms, of which the two largest ones (E1A 289R and E1A 243R) execute most of the functions. Smaller isoforms accumulate during the late phase of infection, but their function is less well understood.[35,36] Owing to its central role in the infection cycle *E1A* constitutes a conceivable target for CRISPR/Cas9-based inhibition of adenovirus replication.

In this study, we provide a proof-of-principle that adenoviral DNA is amenable to recognition by CRISPR/Cas9, and that the time-frame during which the viral DNA is accessible is large enough to allow for efficient inhibition of virus replication by up to three orders of magnitude when the viral *E1A* gene is targeted. Our data suggest that CRISPR/Cas9 has the intrinsic ability to cope with the sheer numbers of viral DNA molecules generated during lytic adenovirus infection, provided that efficient delivery/production of CRISPR/Cas9 effectors is ensured.

RESULTS

Selection of adenovirus-targeting gRNAs and delivery of CRISPR/Cas9 effectors

To evaluate the potential of CRISPR/Cas9 to inhibit the multiplication of lytically replicating adenoviruses, we chose HAdV-5 as a model system and the E1A gene as the target. We selected 10 gRNAs predicted to bind to the left half of *E1A* (Figure 1), comprising the functionally important conserved regions 1 and 2, both of which are part of the dominating E1A isoforms E1A 289R and E1A 243R. As a Cas9 effector, we used a high-fidelity version of Cas9, spCas9-HF1, which has greatly reduced off-target cleavage activity.[37] For delivery, we employed replication-deficient, E1- and E3-deleted, HAdV-5-based vectors as these vectors ensure efficient delivery into target cells *in vitro* and are amplified in cells infected with HAdV-5, thereby increasing the copy number of CRISPR/Cas9 effector-encoding sequences in these cells. We generated vectors containing the expression cassettes for Cas9 alone and Cas9 together with individual targeting gRNAs or a non-targeting control gRNA (Figure 2). These vectors need to be amplified in HEK 293 cells expressing adenoviral *E1A* for complementation of *E1A* deletion in the vectors. To avoid targeting HEK 293-encoded E1A during vector amplification, Cas9 was placed under the control of a tetracycline-regulatable CMV promoter, and vectors were amplified in T-REx-293 cells, a derivative of HEK 293 cells stably expressing the tetracycline repressor. Western blot analysis confirmed the expression of Cas9 (Figure S1). The gRNA sequences were transcribed from the human U6 promoter (Figure 2).

Cas9 cleavage reporter assays reveal functional, E1A-targeting, gRNAs

To evaluate the functionality of the gRNAs, DNA from HeLa cells transduced with Cas9/gRNA expression vectors and infected with HAdV-5 was subjected to T7 endonuclease mismatch assays. Briefly, PCR amplicons of the region spanning the target sites were denatured, reannealed, and treated with T7 endonuclease to allow for the cleavage of mismatches arising after annealing of altered and unaltered target sites. Agarose gel electrophoresis identified cleavage products for gRNAs 1, 2, 7, 8, and 9 (Figure 3A), indicating that these gRNAs were capable of cleaving their targets. The functionality of two of these gRNAs selected for further experiments (gRNAs 8 and 9) was further

validated in a surrogate reporter system based on the expression of an RFP-eGFP fusion protein.[38] Briefly, we inserted the *E1A* target sequences between an RFP-encoding sequence and two out-of-frame eGFP-encoding sequences, giving rise to a fusion protein that was only active for RFP but not for eGFP (Figure 3B). Upon insertion of indels into the target site by CRISPR/Cas9, the generated frameshift mutations were expected to render eGFP in frame with RFP, resulting in both red and green fluorescence. Microscopy revealed the appearance of such cells in the presence of the Cas9/gRNA vectors (Figure 3C), indicating the functionality of the gRNAs. No green-fluorescent cells appeared upon treatment with vectors expressing a non-targeting gRNA or Cas9 alone.

CRISPR/Cas9 decreases the generation of infectious viral particles and of viral DNA

To test whether gRNAs 8 and 9 were capable of inhibiting the replication of HAdV-5, we transduced HeLa cells with the respective adenoviral vectors or negative control vectors and infected them with HAdV-5 24 h after transduction. Two days after the infection, the number of infectious virus particles was determined. As shown in Figure 4A, gRNAs 8 and 9 decreased infectious virus particle numbers by one order of magnitude (90.4%) and 1.9 orders of magnitude (98.8%), respectively, compared to the non-targeting control gRNA. To examine whether the inhibition can be maintained over a longer period of time, we conducted infection experiments over a period of 6 days and observed a decrease in the number of infectious virus progeny at all time points. At the latest time point, infectious HAdV-5 virus progeny was reduced by 0.6 orders of magnitude (75%) and 0.7 orders of magnitude (80%), respectively, compared to the non-targeting gRNA. CRISPR/Cas9 also decreased viral genome copy numbers: viral DNA was reduced by 0.87 orders of magnitude (86.5%) and 1.6 orders of magnitude (97.6%) by gRNAs 8 and 9, respectively. As E1A activity was obviously not completely abrogated by CRISPR/Cas9 the otherwise replication-deficient, *E1A*-lacking, vectors were also replicated in those cells (Figure S2). The amplification of the vectors was highest in the presence of the non-targeting gRNA while it was reduced when E1A-targeting gRNAs were expressed.

A combination of CRISPR/Cas9 and CDV leads to increased inhibition of virus replication

As we expected high target DNA numbers to represent a factor that would limit the degree of inhibition by CRISPR/Cas9 and to potentially enhance the CRISPR/Cas9-mediated effect, we additionally treated the cells for 6 days with the viral DNA synthesis-inhibiting nucleoside analog CDV at concentrations of 10 and 30 µM. CDV at 30 µM reflects *in vivo* peak serum levels typically achieved after intravenous administration of CDV.[39] The CRISPR/Cas9 approach alone decreased the number of infectious virus particles by approximately one order of magnitude at all time points (Figure 5A). On day 2 post-infection, the higher CDV concentration of 30 µM led to a comparable reduction in the number of infectious virus particles. However, at later time points, treatment with 30 µM CDV decreased the output of infectious virus particles more efficiently than CRISPR/Cas9. The combination did not reveal an additive effect at this CDV concentration, probably due to the already very high inhibitory effect exerted by CDV alone. However, at the lower concentration of 10 µM, CDV alone showed a similar degree of inhibition compared to CRISPR/Cas9, and a combination of both led to a pronounced additive effect at all later time points. At the latest time point, the combination of CRISPR/Cas9 and CDV resulted in an additive effect of 2.76 and 2.97 orders of magnitude in comparison to the sole inhibitory effects of CRISPR/Cas9 and CDV, respectively (Figure 5B), culminating in a total reduction of infectious virus particles by 3.47 orders of magnitude (99,97%) compared to the treatment with the non-targeting gRNA in the absence of CDV.

A combination of four gRNAs enhances the inhibition of virus replication

Targeting a virus with more than one gRNA is not only beneficial for the overall inhibitory effect; it is also mandatory as the introduction of indels that do not change the reading frame by a single gRNA would inevitably generate escape mutants that could no longer be targeted by the initial gRNA, consequently rendering them resistant. Thus, the expressions of four gRNAs were combined. We combined the previously evaluated gRNAs 8 and 9 with gRNAs 1 and 7. Similar to gRNAs 8 and 9, the functionality of gRNAs 1 and 7 was in addition to the assessment in T7 endonuclease assays, which had indicated functionality (Figure 3A) also proven in the eGFP reporter system (Figure S3). The four gRNAs were expressed in four cassettes, each containing an individual RNA polymerase III promoter

(Figure 6A). A control vector expressing four identical non-targeting gRNAs was also generated. The capacity of the 4-gRNA vector to inhibit HAdV-5 replication was assessed over a period of six days (Figure 6B). The degree of inhibition was significantly more pronounced than that achieved with vectors containing only one gRNA. While the virus concentration in cells treated with the non-targeting gRNAs construct increased to between 1e+05 and 1e+06 infectious particles per mL, it remained around 1e+02 to 1e+03 infectious particles per mL in cells treated with the targeting gRNAs construct. At the latest time point, *E1A*-targeting reduced infectious virus progeny by 2.8 orders of magnitude (99.8%) compared to the non-targeting control.

To test whether this vector was also potent enough to inhibit HAdV-5 replication when applied shortly after infection, HeLa cells were first infected with HAdV-5, followed by transduction with the vectors 6 h post-infection. As secondary infection events taking place in the cultures at later time points would occur in the presence of already established high levels of Cas9 and gRNAs, thereby no longer representing conditions where Cas9 and gRNA levels have to be built up first, infectious virus particle numbers were only determined 48 h post-infection. Indeed, inhibition of HAdV-5 replication was also strong under these conditions (Figure 6C), suggesting that the buildup of sufficiently high levels of CRISPR effectors must be rapid. The inhibition of HAdV-5 replication by CRISPR/Cas9 was in general not restricted to HeLa cells but occurred in other cells such as A549 cells as well (Figure S4). Moreover, it was also observed when we increased the number of viral targets relative to vector copy numbers by raising the MOI for HAdV-5 to up to 50 to generate a more realistic scenario in which one vector molecule encounters one wild-type virus per cell (1:1 ratio) (Figure S5).

Since the 4-gRNA expression cassette is relatively large, and given the fact that the cloning space in certain delivery vectors (*e.g.*, AAV vectors) is limited, we thought to generate an alternative, space-saving, expression cassette (Figure 7A). In this cassette, the four targeting or non-targeting gRNAs are separated by artificial, non-targeting, pri-miRNA sequences based on the murine mmu-miR-155 pri-miRNA scaffold[40] and are transcribed as a polycistronic gRNA-miRNA precursor from a single RNA polymerase III promoter. Individual gRNAs are liberated from this precursor by DROSHA, which cleaves pri-miRNAs at a specific site at the base of each hairpin.[41,42] The exact DROSHA cleavage site

for mmu-miR-155 has been previously determined, and the mmu-miR-155 scaffold has been developed into an amiRNA expression system.[43] Approaches for multiplex expression of gRNAs from polycistronic gRNA-amiRNA precursors have been proven to be functional in other contexts.[44,45] The final constructs were tested for their ability to inhibit HAdV-5 replication as before, and the two different vector types for multiplex gRNA expression were also evaluated side-by-side: a comparison of the inhibition of HAdV-5 replication by the vectors containing four separate expression cassettes, as depicted in Figure 6B, with that by the vectors expressing the gRNAs from a common promoter that was tested in parallel is shown in Figure 7B. Over a period of six days, the degree of inhibition exerted by the two vector types was very similar. At 6 days post-infection, the vector expressing the gRNAs from a single promoter also drastically reduced the output of infectious virus progeny (by three orders of magnitude) and it was also able to decrease the number of infectious virus particles when applied to already infected HeLa cells 6 h post-infection (Figure 7C).

Next generation sequencing-based analysis of CRISPR/Cas9-induced mutations

To confirm the introduction of mutations into *E1A*, DNA was isolated from HAdV-5 infected cells that had been transduced with the adenoviral CRISPR/Cas9 vector expressing the four gRNAs from individual promoters or from cells that had only been infected with HAdV-5 (background control). The target region was amplified by PCR and the amplified DNA was sequenced. Of 1179 reads mapped to the amplicon sequence, 83.89% harbored deletions, insertions, or inversions (Figure 8A). When combining two or more gRNAs, the generation of deletions between target sites is expected to be the most frequent alteration. Indeed, sequencing indicated that deletions between target sites were the most dominant type of mutations. Small indels at the cleavage sites were almost negligible. We found deletions between all target sites, the most frequent of which were those between the target sites for gRNAs 9 and 1, while the least frequent were those between gRNA 8 and other target sites. All other deletions ranged in-between. The region comprising the target site for gRNA 7 was most heavily affected by the mutations, as it was either completely deleted (in 57.3% of all reads mapped to the amplicon region) or showed deletions going leftward or rightward towards the

adjacent target sites. Figure 8B shows a multiple sequence alignment of mutated sequences mapped to this region. Additionally, but to a lesser extent, we identified inversions between target sites and duplications in some cases coupled with deletions. The identified mutations are shown in Figure 8C. Almost all deletions and inversions led to frameshifts, and consequently to truncated E1A proteins lacking at least CR2, CR3, and CR4, and in most cases, most of the linker between CR1 and CR2 (Figure 8D). For some of the mutations depicted in Figure 8C, a few more variants existed that differed in harboring or lacking an additional single nucleotide at the cleavage site of gRNA 9 in certain types of junctions. Importantly, however, all of these variants, showed frame shifts starting at the cleavage site of gRNA 9 and resulted in truncated proteins with out-of-frame sequences from amino acids 74 or 75 onward. This pattern occurred in all these cases, regardless of which other types of mutations in regions further downstream had additionally taken place. We found one case in which a specific joining of the fragments caused the amino acid sequence to jump back into the original reading frame at a position further downstream (indicated as del 9-7 [2] in Figure 8C). This type of joining is caused by either offset cleavage by one nucleotide or, more likely, by removal of an end-standing nucleotide prior to the repair of the gap. However, this specific mutation constituted <1% of all deletions, inversions, and duplications in which gRNA 9 was involved. In general, we observed cleavage only at unique positions. No small indels or larger deletions, insertions, or duplications were found in the DNA isolated from cells infected with HAdV-5 in the absence of Cas9. The data indicated that ~16% of E1A sequences represented wild-type E1A, likely originating from viruses that had not been reached by CRISPR/Cas9. Accordingly, virus recovered after targeting was able to replicate and was still amenable to CRISPR/Cas9-mediated inhibition in a subsequent round of targeting (Figure S6). An evaluation of the specificity of SpCas9-HF1 targeting did not reveal cleavage at sites with some potential for off-target cleavage (Figure S7).

DISCUSSION

Our data indicated that replicating adenoviral DNA is accessible to CRISPR/Cas9, as targeting of *E1A* can lead to a decrease in infectious virus progeny. Moreover, the time window during which

CRISPR/Cas9 effectors have access to the viral DNA before it is encapsidated, seems to be large enough to allow for effective cleavage. The rationale for choosing *E1A* as a target was based on our previous data, which revealed that RNAi-mediated reduction of only certain early viral gene products that directly (pTP, viral DNA polymerase) or indirectly (E1A) affect viral DNA replication leads to a decrease of infectious virus progeny.[46] Preventing the accumulation of high numbers of target molecules that would occur once viral DNA replication started seems to have been the key to these experiments and is probably also required in CRISPR/Cas9-based approaches. We expected particularly *E1A* to be a promising target, as it is required for the expression of other adenoviral genes, and its knockout would consequently render mutant viruses barely active. In contrast, pTP or DNA polymerase mutants would also be replication-deficient but would still produce functional E1A proteins with multiple consequences for various cellular and viral processes.

Cleavage of the target DNA obviously occurred soon enough after infection to exert a negative effect on viral replication. This effect may be due to the impairment of E1A-mediated transcriptional activation of other viral genes. We previously observed that the knockdown of *E1A* expression by RNAi had an immediate negative effect on the expression of later viral genes involved in viral DNA replication and consequently on viral DNA copy numbers.[46] Knockout of E1A by CRISPR/Cas9 probably caused the same effect. In addition, it is likely that cleavage of viral DNA directly reduced viral genome copy numbers and hindered the synthesis of full-length viral DNA. Together, these effects probably ensured a reasonable ratio between the target molecules and CRISPR/Cas9 effectors. Treatment with low concentrations of CDV seemed to have shifted this ratio further in favor of CRISPR/Cas9 effectors, leading to even higher degrees of inhibition (Figure 5B). The time window during which viral DNA is accessible becomes even smaller when trying to target infected cells. However, the accumulation of gRNA and Cas9 effectors seemed to have occurred quickly enough to efficiently cleave the target DNA, even in those cells (Figures 6C and 7C). It is possible that the replication of the adenoviral vectors which was triggered by HAdV-5 at least to some extent even in the presence of *E1A*-targeting gRNAs (Figure S2) contributed to building up sufficiently high levels in a short time. We had also proven this beneficial amplification effect when targeting HAdV-5 mRNA

with adenoviral vector-expressed artificial miRNAs.[47] Such systems are self-balancing in so far as vector will be generated as long as the inhibition of wild-type virus activity is not complete which may generate a supply of vector at sites of infection *in vivo*. Adenoviral vectors and to an even higher extent adeno-associated viral vectors (AAV vectors)[48] are among the most promising viral vectors for gene delivery *in vivo* and are conceivable delivery vehicles for any CRISPR/Cas9-based application. AAV vectors are also amplified in the presence of wild-type adenovirus.[49] However, the use of AAV vectors is often hampered by their limited cloning capacity, which prevents the insertion of larger fragments. This also applies to the multi-gRNA expression cassettes in which each gRNA is expressed from individual promoters. The fact that our much shorter, polycistronic gRNA/pri-amiRNA expression cassette performed equally well opens up the possibility of its incorporation into AAV vectors to permit efficient *in vivo* delivery and amplification in adenovirus-infected cells. Moreover, this design allows to incorporate a higher number of gRNAs in a space-saving manner to simultaneously target a wider range of adenovirus serotypes such as – just to mention one conceivable application – the whole spectrum of serotypes associated with ocular infections.

Targeting of *E1A* with four different gRNAs caused a variety of deletions, inversions, or duplications (Figure 8), of which the majority (>99%) caused frameshifts rendering E1A defective in functions residing in CR2, CR3, CR4, and when the target site for gRNA8 was involved, also CR1. There are numerous consequences to E1A function. To give but one example, the lack of CR3, which is important for the transactivation of other early adenoviral promoters[50,51] is expected to result in a strong inhibitory effect on viral DNA replication, as the expression of viral E2 genes encoding the proteins for viral DNA synthesis is dependent on the transactivation mediated by CR3. E1A functions residing in other conserved regions affected by the mutations are extremely heterogeneous and affect processes such as further activation of viral and cellular transcription, de-repression of transcription, cell cycle entry control, differentiation, stabilization of p53, immortalization of cells, apoptosis, or proteasomal degradation[30,31] all of which contribute to viral replication.

E1A exerts most of its functions in the nucleus[31] and a lack of its import into the nucleus has severe consequences on E1A function.[52,53] All frameshift mutations removed the canonical nuclear

294 localization signal (NLS) at the C-terminus of E1A (aa 258-263 and 285-288) whose deletion impairs
295 nuclear localization[54-56] and a second noncanonical NLS located within CR3 (aa 142-182).[57,58] A
296 sequence in the N-terminal/CR1 part of E1A (aa 30-69) that seems to contribute to the nuclear
297 localization of E1A, albeit to a lesser extent,[57] was only removed by the largest deletions involving
298 cleavage by gRNA8. Thus, the observed mutations probably had a more general impact by negatively
299 affecting the subcellular localization of E1A, with consequences for its activity, regardless of which
300 other functions of the protein had been directly eliminated by the mutations. Together, the loss of
301 functions directly associated with the mutated regions and the impairment of the proper localization
302 of the E1A remnants may explain the pronounced inhibitory effect on virus multiplication.
303 The mutation frequency of approximately 84% translated into a 2.5 to 2.8 orders of magnitude
304 decrease in infectious virus progeny. This is about the same degree of inhibition that we had
305 previously observed when targeting HAdV-5 with siRNAs or amiRNAs.[46,47] Since potent siRNAs and
306 amiRNAs typically reduce target RNA levels by 80% to 90% and assuming in a very simplistic way that
307 the respective protein levels become decreased to a similar extent, the degree of inhibition reported
308 here reflects what we had previously observed when targeting adenoviral RNA. Thus, CRISPR/Cas9-
309 based methods appear to be capable of decreasing infectious virus progeny *in vitro* to approximately
310 the same extent as RNAi-based methods. As amiRNA-based methods have been shown to also inhibit
311 adenovirus replication *in vivo*,[59] CRISPR/Cas9 may have similar potential. It was shown that AAV
312 vector-mediated amiRNA delivery inhibited HAdV-5 replication not only locally in the mainly target
313 organ (the liver) but led to a general decrease of virus load in the animals,[59] demonstrating that
314 delivering anti-adenoviral effectors to the liver, which functions as a virus multiplicator, is a
315 promising strategy to decrease overall virus load in systemically infected animals. Analogously, by
316 applying similar delivery strategies, CRISPR/Cas9 may have a chance to be developed into a
317 therapeutic tool to treat systemic infections. However, due to risks associated with any systemic
318 delivery, topical treatment of localized infections would probably have a higher chance for
319 realization. As for example ocular adenovirus infections are typically localized to a confined area they
320 are conceivable to be topically treated with future therapeutics based on CRISPR/Cas9.

MATERIALS AND METHODS

Cell lines and viruses

HEK293 (human embryonic kidney; ATCC CRL-1573), T-REx™-293 (stable integration of tetracycline repressor gene; Thermo Fisher Scientific, R71007), HeLa (human epithelial carcinoma; ATCC CCL-2), and A549 (human epithelial lung carcinoma; ATCC CCL-185) cells were cultivated in Dulbecco's Modified Eagles Medium (DMEM) with stabilized glutamine (Thermo Fisher Scientific, Vienna, Austria) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Vienna, Austria) in a humidified 5% CO_2 atmosphere at 37°C.

HAdV-5 (ATCC VR-5) was amplified in HEK293 cells; recombinant adenoviral vectors were amplified in T-REx™-293 cells in the absence of doxycycline. Wild-type virus and recombinant vectors were purified by CsCl centrifugation or with a Fast Trap Adenovirus Purification and Concentration Kit™ (Merck/Millipore, Vienna, Austria). Titers of HAdV-5 were determined on HeLa cells with an Adeno-X™ Rapid Titer Kit according to the instructions of the manufacturer (Takara Bio Europe, Paris, France); titers of recombinant HAdV-5 were analogously determined on T-REx™-293 cells in the absence of doxycycline.

CRISPR/Cas9 expression vectors

Vector designs and sequence analyses were performed in CLC Main Workbench 8.1.2. (QIAGEN, Hilden, Germany). To construct the individual Cas9/gRNA expression vectors the SpCas9-HF1 coding sequence[37] was amplified by PCR from plasmid VP12 (obtained from Addgene) with primers Cas9 HF1 FW and Cas9 HF1 RV and the fragment was cloned into the *Eco*RI and *Not*I sites of pENTR4 (Thermo Fisher Scientific, Vienna, Austria). The tetracycline-regulatable CMV-tetO2 promoter was amplified by PCR from pcDNA6.2-GW/EmGFP-miR-luc (Thermo Fisher, Vienna, Austria) with primers CMV Tet02_FW and CMV Tet02_FW and the resulting fragment was cloned into the *Nco*I and *Eco*RI sites of

the same vector. For the amplification and sequencing of pENTR4-based vector primers pENTR4 FW and RV were used.

The selection of the gRNAs (Figure 2B) and the estimation of the targeting probabilities was performed with CHOP-CHOP[60], CasFinder (2017; available online: http://arep.med.harvard.edu/CasFinder/), and CRISPOR (2020; available online: http://crispor.tefor.net/). The gRNAs were designed as described[61] to minimize off-target effects. Linear DNA fragments (gBLOCKS™) containing individual targeting or non-targeting gRNAs under control of a human U6 promoter[62] were generated by gene synthesis (Integrated DNA Technologies, Coralville, USA) and the individual expression cassettes were inserted into the *Hind*II and *NotI* sites of the SpCas9-HF1 expression cassette-containing intermediate vector giving rise to vectors pENTR-CMV-TetO2-spCas9-HF1-hU6-gRNA 1 to pENTR- CMV-TetO2-spCas9-HF1-hU6-gRNA 10 containing a single targeting gRNA, each, and to the control vectors containing non-targeting gRNAs (pENTR-CMV-TetO2-spCas9-HF1-hU6-NT gRNA) or containing only the spCas9-HF1 expression cassette (pENTR-CMV-TetO2-spCas9-HF1).

For the construction of the plasmid vectors containing 4 targeting or non-targeting gRNAs, each expressed from its own promoter (4 x gRNA-E1A, 4x gRNA-NT), the individual expression cassettes were generated by gene synthesis (Biomatik, Kitchener, Canada) and the fragments were transferred into the *Hind*II and *Not*I sites of the CMV-TetO2-spCas9-HF1 backbone. Vectors 4 x (gRNA-E1A / amiRNA) and 4 x (gRNA-NT / amiRNA) containing an array of 4 targeting or non-targeting gRNAs separated by non-targeting amiRNA hairpin sequences (originating from pcDNA6.2-GW/EmGFP-miR-neg; Thermo Fisher, Vienna, Austria) were constructed in an analogous way.

The entire expression cassettes present in the plasmid vectors were eventually moved into the deleted E1 region of the adenoviral vector pAd/PL-DEST (Thermo Fisher, Vienna, Austria) by employing the Gateway™ system for site-specific recombination between sequences flanking the cassettes and the corresponding sequences located on the adenoviral vector. The resulting adenoviral vectors were named Ad E1A-gRNA 1 to Ad E1A-gRNA 10 (containing the individual targeting gRNAs 1 to 10), Ad NT-gRNA (containing a non-targeting gRNA), Ad 4 x gRNA-E1A and Ad 4

x gRNA-NT (containing 4 targeting and 4 non-targeting gRNAs, respectively, expressed from individual promoters), Ad 4 x (gRNA-E1A / amiRNA) and 4 x (gRNA-NT / amiRNA) (containing arrays of 4 targeting and 4 non-targeting gRNAs, respectively), and Ad Cas9 (containing only spCas9-HF1).

Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs, Frankfurt am Main, Germany). PCR reactions were performed with Quick-Load® *Taq* 2X Master Mix (New England Biolabs, Frankfurt am Main, Germany) and Q5® High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt am Main, Germany). Plasmid DNA was extracted with a QIAprep Mini or Midi kit and PCR products were purified with a QIAquick PCR purification kit. All kits for nucleic acid purification were acquired from QIAGEN (Hilden, Germany). All primers are listed in Table S1.

T7 endonuclease mismatch assay

1.5e+04 HeLa cells seeded into the wells of a 96 well plate were transduced with adenoviral vectors expressing Cas9 and individual gRNAs at an MOI of 100 followed by infection with HAdV-5 at an MOI of 0,01 24h post-transduction. At day 4 after infection with HAdV-5 DNA was isolated and three nested PCR reactions using a Q5 high-fidelity DNA polymerase PCR system (New England Biolabs, Frankfurt am Main, Germany) and primer pairs T7E1 E1A Set1 to 3 (Table S1), were performed. Amplified DNA was heat-denatured and allowed to re-anneal to form heteroduplexes between altered and unaltered single strands. The samples subsequently were purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), digested with T7 Endonuclease 1 (New England Biolabs, Frankfurt am Main, Germany) and analyzed by agarose gel electrophoresis.

EGFP reporter system

To detect cells with nuclease induced mutations and test the functionality of the gRNAs a surrogate reporter system (Figure 3B) was adopted.[38] Briefly, double-stranded, linear oligonucleotides representing E1A target regions each carrying clusters of 2 to 3 individual target sites in close proximity to each other were inserted into the *Eco*R1 and *Bam*HI sites of vector RVO1 (PNA Bio, Newbury Park, CA, USA) giving rise to vectors RV01-1,3,4 (carrying the target sites for

gRNAs 1,3 and 4), RV01-2,9 (carrying the target sites for gRNAs 2 and 9), RV01-5,6,8 (carrying the target sites for gRNAs 5,6 and 8) and RV01-7,10 (carrying the target sites for gRNAs 7 and 10). The final RV01-based vectors were sequenced using primers RV01 sequencing FW and RV (Supplementary Table 1). The inserted target sequences are listed in Table S2. 2e+04 to 5e+04 HeLa cells were seeded into the wells of a 96-well plate and were transfected with 100 ng of reporter vectors using Lipofectamine 2000 (Thermo Fisher Scientific, Vienna, Austria) followed by transduction with the recombinant, Cas9/gRNA-expressing vectors. At 48 h post-transduction pictures were acquired with a Leica DMi8 System and were analyzed with the microscope software platform LAS X Life Science (Leica, Wetzlar, Germany).

Western blotting

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on Mini-Protean TGX Precast protein gels (Bio-Rad, Hercules, CA,USA) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) using a Turbo transfer system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% BSA T-BST (500mM Tris HCl pH7.5, 1.5 M NaCl, 0,05% Tween 20). SpCas9-HF1 and β-actin were detected with antibodies 7A9-3A3 (Cell Signaling Technology, Danvers, MA, USA) and GTX629630-25 (GeneTex, Irvine, CA, USA), respectively. Membranes were probed with fluorescent secondary antibodies IRDye® 800CW Goat anti-Mouse (925-32210; LI-COR, Lincoln, NE, USA) and IRDye® 680RD Goat anti-Rabbit (925-68071; LI-COR, Lincoln, NE, USA), respectively, and bands were visualized with a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Virus inhibition experiments

For prophylactic inhibition of virus replication 1.5e+04 HeLa or A549 cells were seeded into the wells of a 96-well plate and were transduced with the recombinant adenoviruses at an MOI of 100. 24 h later cells were infected with HAdV-5 at an MOI of 0.01. In an alternative approach, cells were first infected with HAdV-5 at an MOI of 0.01 followed by transduction with the adenoviral vectors 6 h after infection. For virus inhibition experiments at higher HAdV-5 MOIs A549 cells were seeded into

the wells of a 96-well plate as before and were transduced with the recombinant adenoviruses at an MOI of 50 followed by infection with HAdV-5 24 h later at MOIs ranging from 0.05 to 50.

Determination of HAdV-5 and adenoviral vector DNA copy numbers

DNA was isolated from crude lysates with a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and HAdV-5 DNA was quantified by qPCR using a TaqMan primer/probe set (Supplementary Table 1) specific for the adenoviral E3 gene. Adenovirus genome copy numbers were calculated by serial dilutions of an adenoviral reference DNA. qPCRs conditions were: 1 x (50°C-30sec, 95°C-3min); 40x (95°C-10sec, 60°C-30sec). Adenoviral vector DNA was quantified analogously with a primer/probe set specific for the Cas9-encoding part of the vectors.

Sequencing

For LoopSeq™ [63] adenoviral DNA isolated from cells transduced with recombinant adenovirus and infected with HAdV-5 or only infected with HAdV-5 was subjected to PCR to amplify a segment in the left end of HAdV-5 containing all individual target sites. Amplification was performed with primers E1A/E1B loop seq FW and E1A/E1B loop seq RV (Table S1) and a Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs, Frankfurt am Main, Germany). The amplicon sequences were determined at Loop Genomics (San Jose, CA, USA). FASTQ data were analyzed via CRISPResso2[64] for quantification and location of specific CRISPR/Cas9-induced mutations. Potential off-target cleavage was analyzed by amplifying the respective regions with the primers specified in Table S1 and amplicon sequences were determined at Eurofins (Ebersberg, Germany). FASTQ data were analyzed with CRISPResso2.[64]

Statistical analysis

GraphPad Prism version 8.00 was used to analyze and graph the data. All data are expressed as mean ± standard deviation (SD). To test for statistical significance in the inhibition experiments one-

451 way or two-way ANOVA was used. For multiple comparisons the Dunnett´s test was employed.

452 Statistical significance is indicated for each experiment (*p< 0.05; **p<0.01; ***p<0.001).

SUPPLEMENTAL INFORMATION

453 Supplemental Information includes two figures and two tables and can be found with this article

454 online at…

AUTHOR CONTRIBUTIONS

455 Z.D., F.R., A.W., A.J., K.Z. and R.K. were responsible for the conception of the work and the design of

456 the experiments. Z.D., F.R., K.Z., A.J., I.K. and R.K. performed the experiments. A.W. and R.K. analyzed

457 the data. Z.D., F.R., A.J., K.Z. and R.K. wrote the paper. A.W. and I.K. revised the work.

CONFLICTS OF INTEREST

458 The authors declare no conflicts of interest.

DATA AVAILABILITY

459 The most relevant datasets generated and analyzed as part of this study are included in this

460 published article and its supplementary information files. Raw data analyzed in the study are

461 available from the corresponding author on request.

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KEYWORDS

463 CRISPR/Cas9, adenovirus, infection, virus, therapy

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FIGURE LEGENDS

Figure 1. Binding sites of E1A-targeting gRNAs.

The E1A gene serving as the target for CRISPR/Cas9-mediated cleavage of the viral DNA is shown in detail. The gRNAs binding to E1A are indicated by arrows numbered from 1 to 10. The orientation of the arrows indicates binding to the plus and the minus strand, respectively. The binding sites within the two most important E1A isoforms (243R and 289R) are depicted.

Figure 2. Structure of the recombinant adenovirus vector constructs and gRNA sequences incorporated into them.

A. Schematic representation of HAdV-5-based vectors with inserted individual gRNA sequences. All adenoviral vectors are based on the HAdV-5-derived vector pAd/PL-DEST ™ (Thermo Fisher Scientific) and lack the E1 and E3 regions. Cas9 and gRNA expression cassettes were inserted into the deleted E1 region. The expression of spCas9-HF1 is driven by a tetracycline repressor-controlled CMV promoter comprising two binding sites for the repressor (2xTetO2). The expression of the individual targeting or non-targeting gRNAs is under control of a constitutive human U6 (hU6) promoter. The structure and sequence of the gRNAs is exemplarily shown for E1A gRNA 9 bound to its target site. The control vector containing only the Cas9 expression cassette is also depicted. B. Target sequences for the individual gRNAs and their positions within the HAdV-5 genome (AY339865.1).

Figure 3. Detection of CRISPR/Cas9-mediated gene editing in T7 endonuclease mismatch assays and surrogate reporter assays.

A. HeLa cells were transduced with the adenoviral CRISPR/Cas9 expression vectors containing either Cas9 alone or Cas9 in combination with the individual targeting gRNAs or a non-targeting (NT) gRNA at an MOI of 100 followed by infection with HAdV-5 at an MOI of 0.01 24 h later. Four days post-infection DNA was isolated and the target region comprising all target sites was amplified by PCR. The amplicon DNA was heat-denatured, re-annealed to form heteroduplex DNA, subjected to T7

endonuclease I treatment and analyzed by agarose gel electrophoresis. The uncleaved DNA band is indicated with an arrow. Cleavage products indicating heteroduplex DNA formed due to the insertion of mutations by CRISPR/Cas9 at the specific target sites are indicated with asterisks. B. Schematic representation of the eGFP surrogate reporter vectors and of the methodology. A fusion protein of RFP (red) and two out of frame eGFP copies (black), each in a different reading frame, is expressed from a CMV promoter. The E1A region comprising the individual target sites was inserted into the linker region between the RFP and eGFP sequences. Indel formation as a consequence of the repair of DNA double strand breaks generated by CRISPR/Cas9 leads to frameshifts and to the expression of functional eGFP (green) indicating gRNA functionality. RFP expression (red) from the same vectors serves as a transfection control. C. HeLa cells were transfected with the reporter vector and transduced with one of the recombinant adenoviral vectors expressing Cas9 alone or Cas9 in combination with a targeting or a non-targeting (NT) gRNA. Fluorescence was monitored 48 h post-transduction with a Leica DMi8 System. Bright field, red and green fluorescence images at a magnification of 10x are shown. Microscopy settings were: HC PL FLUOTAR CS 10x/0.40 DRY; Camera Leica DFC 360FX: active resolution 1392 x 1040, pixel bitdepth 12/8 bit, pixel size 6.45 µm x 6.45 µm; live image with 1392 x 1040 at 20 images/second.

Figure 4. E1A-targeting gRNAs significantly decrease numbers of infectious viral particles and HAdV-5 genome copy numbers.

A. HeLa cells were transduced with the adenoviral vectors containing either Cas9 alone or Cas9 in combination with the targeting gRNAs 8 or 9 or with a non-targeting (NT) gRNA at an MOI of 100. 24h after transduction cells were infected with HAdV-5 at an MOI of 0.01. Numbers of infectious virus particles were determined at day 2 post-infection and were expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment out of 3. *$p < 0.05$. B. HeLa cells were transduced and infected as in A and were grown for a prolonged period of time. Numbers of infectious virus particles were determined at time points 0, 2, 4, and 6 days post-infection. Data represent the means (n = 3) ± SD of triplicate infections of a

representative experiment out of 3. ****p < 0.0001. C. Same experimental setup as in A with the difference that HAdV-5 genome copy numbers were determined by qPCR. Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment out of 3. *$p < 0.05$.

Figure 5. Low doses of CDV increase the inhibition of HAdV-5 replication by CRISPR/Cas9.
HeLa cells were transduced with the adenoviral CRISPR/Cas9 expression vectors containing either Cas9 alone or Cas9 in combination with E1A gRNA 9 or a non-targeting (NT) gRNA at an MOI of 100 followed by infection with HAdV-5 at an MOI of 0.01 24 h later. Concomitantly with the infection the cultures were treated with or without CDV at a concentration of 30 µM (A) and 10 µM (B), respectively. Numbers of infectious HAdV-5 particles were determined at days 0, 2, 4, and 6 post-infection and were expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment out of 3. ****$p < 0.0001$.

Figure 6. Targeting of E1A with 4 different gRNAs potently inhibits HAdV-5 replication.
A. Schematic representation of the HAdV-5-based CRISPR/Cas9 vectors for multiplex gRNA expression. The sequences of gRNAs 1, 7, 8, and 9 were expressed from individual, constitutive, RNA polymerase III promoters (mU6, h7SK, hH1, and hU6). An analogous vector containing 4 identical, non-targeting, gRNAs instead of the targeting gRNAs was constructed as well. SpCas9-HF1 was expressed from a doxycycline-regulatable CMV promoter harboring two TetO2 binding sites for the tetracycline repressor. B. HeLa cells were transduced with the adenoviral vector containing Cas9 in combination with gRNAs 1, 7, 8, and 9 or with a control vector carrying 4 non-targeting (NT) gRNAs at an MOI of 100 followed by infection of the cells with HAdV-5 at an MOI of 0.01 24 h later. Numbers of infectious virus particles were determined at the indicated time points and were expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment out of 3. *$p < 0.05$; *** $p < 0.001$. C. Experimental settings were as in A with the difference that HeLa cells were infected with HAdV-5 6 hours prior to the transduction with the vectors. Numbers of infectious virus particles were determined at day 2 post-infection and were

expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment out of 3. $^{**}p < 0.01$.

Figure 7. Concatemerized gRNAs separated by non-targeting amiRNA spacers allow potent inhibition of HAdV-5 replication.

A. Schematic representation of the adenoviral vector for multiplex expression of concatemerized gRNAs and the processing of the primary transcripts within the cell. SpCas9-HF1 expression is under control of a doxycycline-regulatable CMV promoter harboring two TetO2 binding sites for the tetracycline repressor and multiplexed gRNAs separated by non-targeting amiRNA are expressed from a common hU6 promoter. A negative control vector carrying 4 non-targeting gRNAs was constructed in an analogous manner. The individual gRNAs are released from the primary transcript by DROSHA-mediated cleavage at the base of each interjacent amiRNA hairpin. The gRNA intermediates are further trimmed by Cas9 to give rise to the mature gRNAs. B. HeLa cells were transduced with the adenoviral vectors containing Cas9 in combination with the concatemerized targeting gRNAs 1, 7, 8 and 9 or with a control vector bearing Cas9 in combination with 4 non-targeting (NT) gRNAs at an MOI of 100. 24h after transduction the cells were infected with HAdV-5 at an MOI of 0.01. Numbers of infectious virus particles were determined at the indicated time points and were expressed as IFU/mL (infectious units per mL). For better comparison the values for the vectors expressing the 4 gRNAs from individual promoters and those for the only-HAdV-5-control presented in Fig. 6D are also shown here as the respective transfection/infection experiments were conducted side-by-side. Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment out of 3. $^{*}p < 0.05$; $^{***}p < 0.001$. C. Experimental settings were as in B with the difference that HeLa cells were infected with HAdV-5 6 hours prior to the transduction with the vectors. Numbers of infectious virus particles were determined at day 2 post-infection and were expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment out of 3. $^{*}p < 0.05$.

Figure 8. Targeting of E1A with 4 different gRNAs primarily leads to deletions, inversions, and duplications in E1A.

HeLa cells were transduced with the adenoviral vectors harboring Cas9 in combination with the 4 gRNAs 1, 7, 8 and 9 expressed from separate promoters at an MOI of 100 or were mock-transduced. 24h after transduction the cells were infected with HAdV-5 at an MOI of 0.01. Two days post-infection DNA was isolated from the cells, a large region comprising all E1A target sites was amplified by PCR, and the amplicon DNA was subjected to loop sequencing. A. Total percentage of deletions/insertions/inversions in the target region. B. Alignment of reads around the most heavily affected region comprising the target site for gRNA 7. Deletions (del; dashed lines), inversions (inv; in red and in lower case), and duplications (dupl; in red and in lower case) are shown. The cleavage position for gRNA 7 is indicated with a vertical dashed line. The frequency of the mutations (percent of reads mapping to the target region) are given right to the alignments. C. Types of deletions, inversions, and duplications detectable in the target region. Deletions are indicated with dashed lines, inversions are colored in yellow. Changed positions of fragments are indicated with arrows. D. Consequences of the mutations for the E1A reading frame. Narrow boxes in grey symbolize the length of the protein. Larger, open, boxes represent the open reading frames. Conserved regions (CR) within the open reading frames are colored. Numbers above the individual schemes indicate the amino acid positions at which the proteins and the open reading frames, respectively, start and end. The starting and end points of the conserved regions are given for comparison.

Figure 1

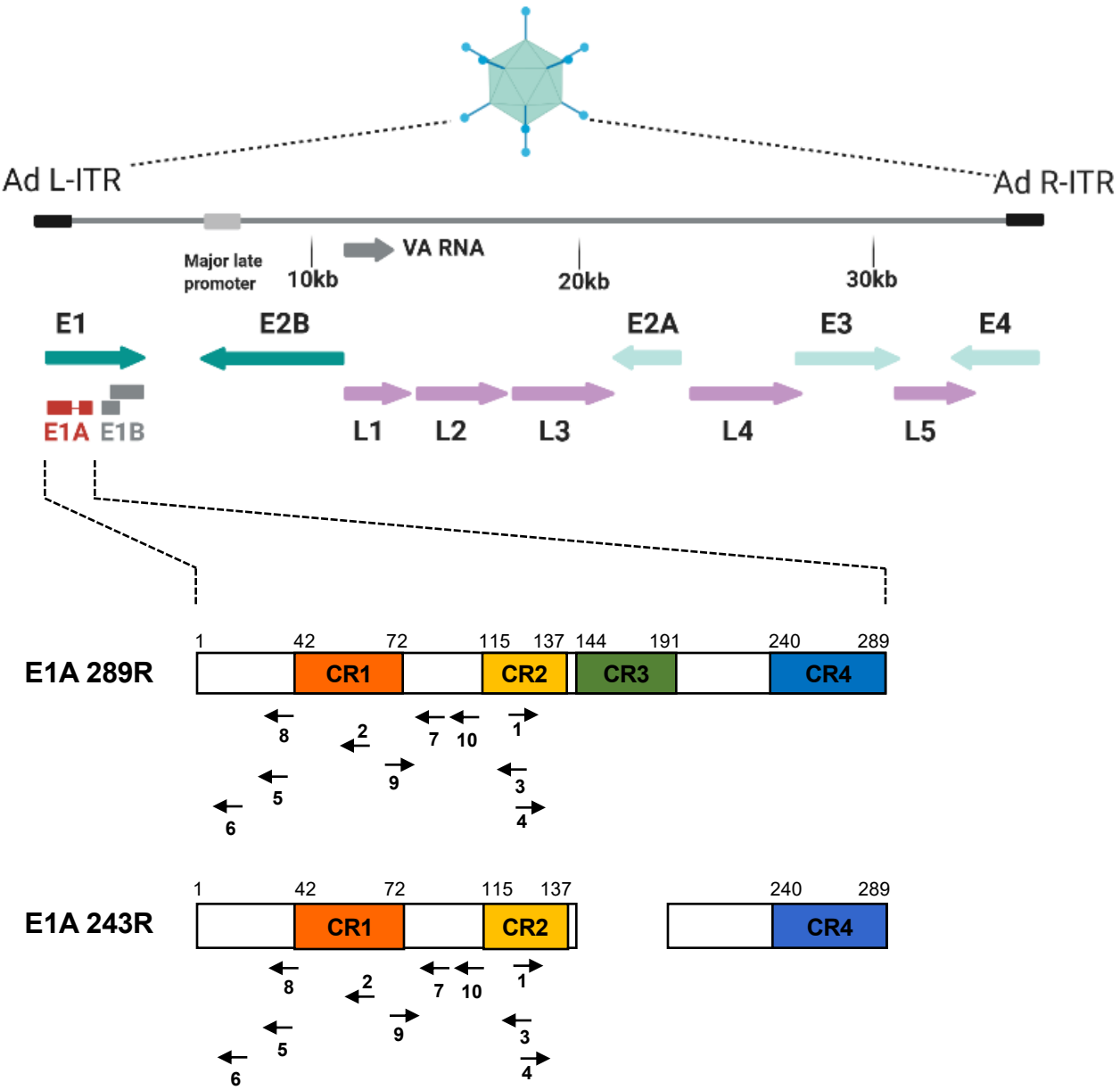
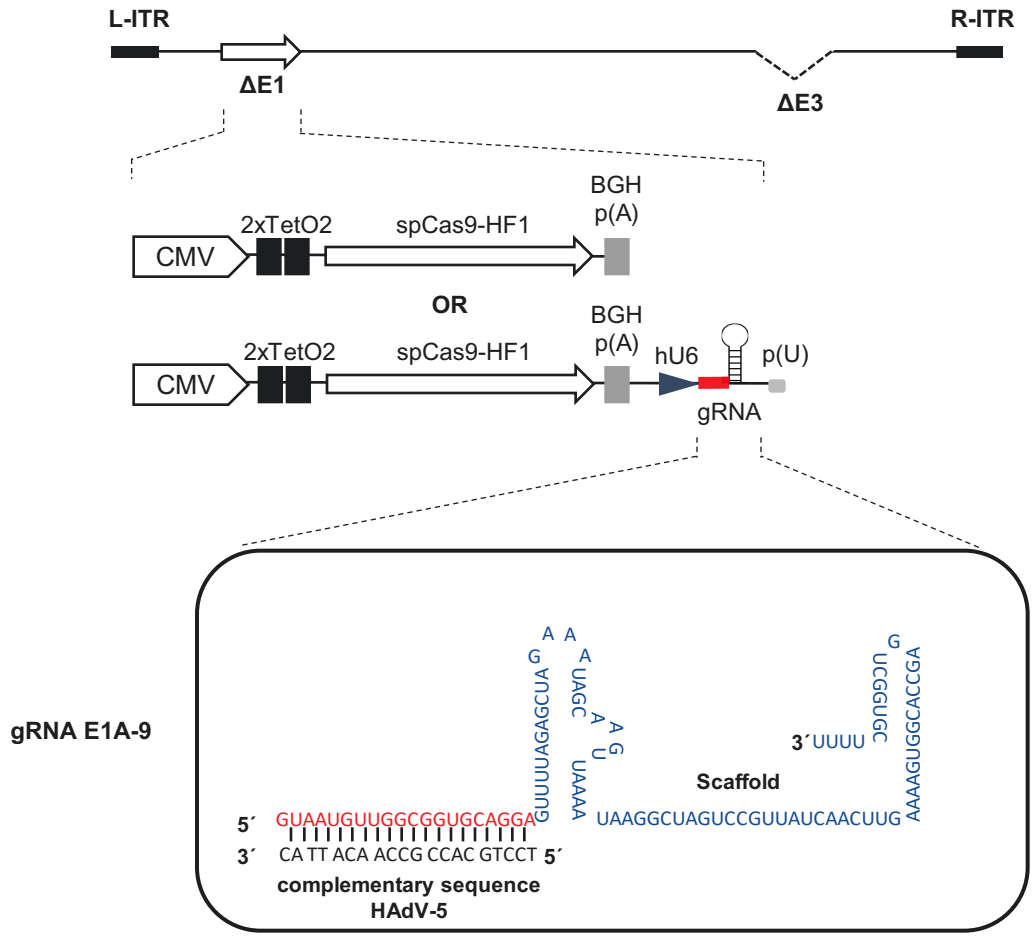

Ad L-ITR
Ad R-ITR
Major late promoter
10kb
VA RNA
20kb
30kb
E1
E2B
E2A
E3
E4
E1A
E1B
L1
L2
L3
L4
L5
E1A 289R
1
42
72
115
137
144
191
240
289
CR1
CR2
CR3
CR4
8
2
7
10
1
9
3
5
6
4
E1A 243R
1
42
72
115
137
240
289
CR1
CR2
CR4
8
2
7
10
1
9
3
5
6
4

Figure 2

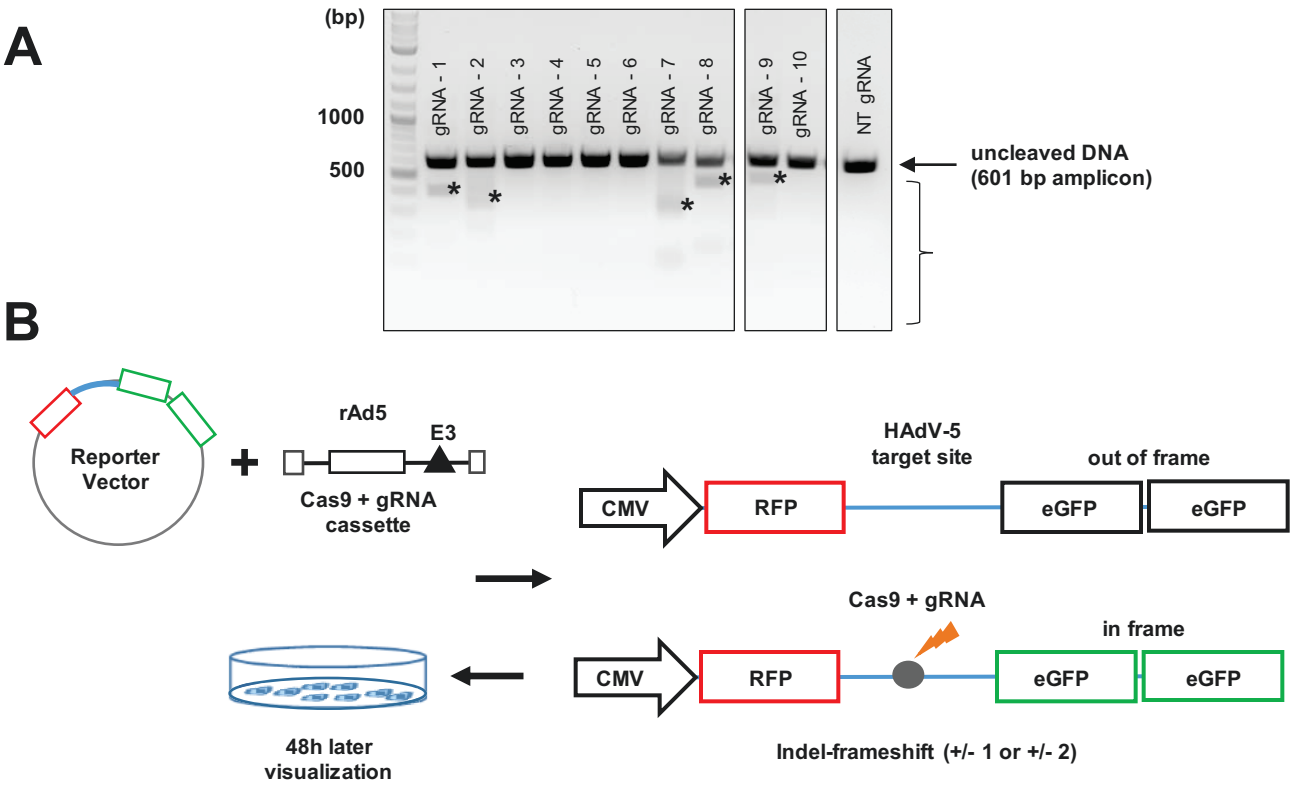
A



B

gRNA name	Target sequence 5`-3`	Target site in HAdV-5 (AY339865.1)
E1A #1	GATCGATCTTACCTGCCACG	916-935
E1A #2	GCCTCCTCGTTGGGATCTTC	722-741
E1A #3	GATCGATCACCTCCGGTACA	904-923
E1A #4	GATCTTACCTGCCACGAGGC	920-939
E1A #5	GGTTCAAAATGGCTAGGAGG	659-678
E1A #6	GATCAGCTGGTCCAAAAGAC	612-631
E1A #7	GTGAGGCGGCTCCGGAGAAC	822-841
E1A #8	GGTGGTTCAAAATGGCTAGG	662-681
E1A #9	GTAATGTTGGCGGTGCAGGA	767-786
E1A #10	GCTGCTCGGGCTGCCGGGAA	844-863
Non-targeting (NT)	GGAGCGATGATACGCGGTGC	

Figure 3


A
(bp)
1000
500
gRNA - 1
gRNA - 2
gRNA - 3
gRNA - 4
gRNA - 5
gRNA - 6
gRNA - 7
gRNA - 8
gRNA - 9
gRNA - 10
NT gRNA
uncleaved DNA
(601 bp amplicon)
B
Reporter Vector
rAd5
E3
Cas9 + gRNA cassette
HAdV-5 target site
out of frame
CMV
RFP
eGFP
eGFP
Cas9 + gRNA
in frame
CMV
RFP
eGFP
eGFP
48h later visualization
Indel-frameshift (+/- 1 or +/- 2)

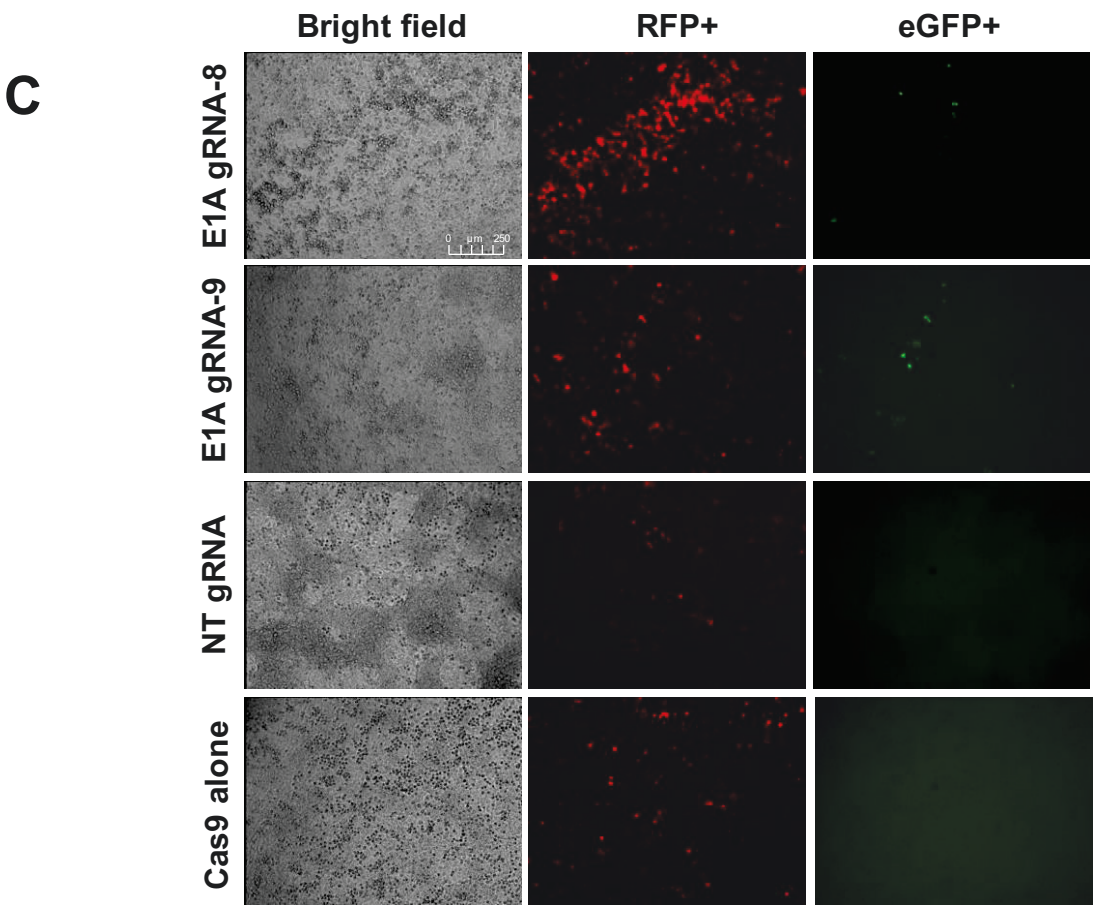

C
Bright field
RFP+
eGFP+
E1A gRNA-8
E1A gRNA-9
NT gRNA
Cas9 alone

Figure 4

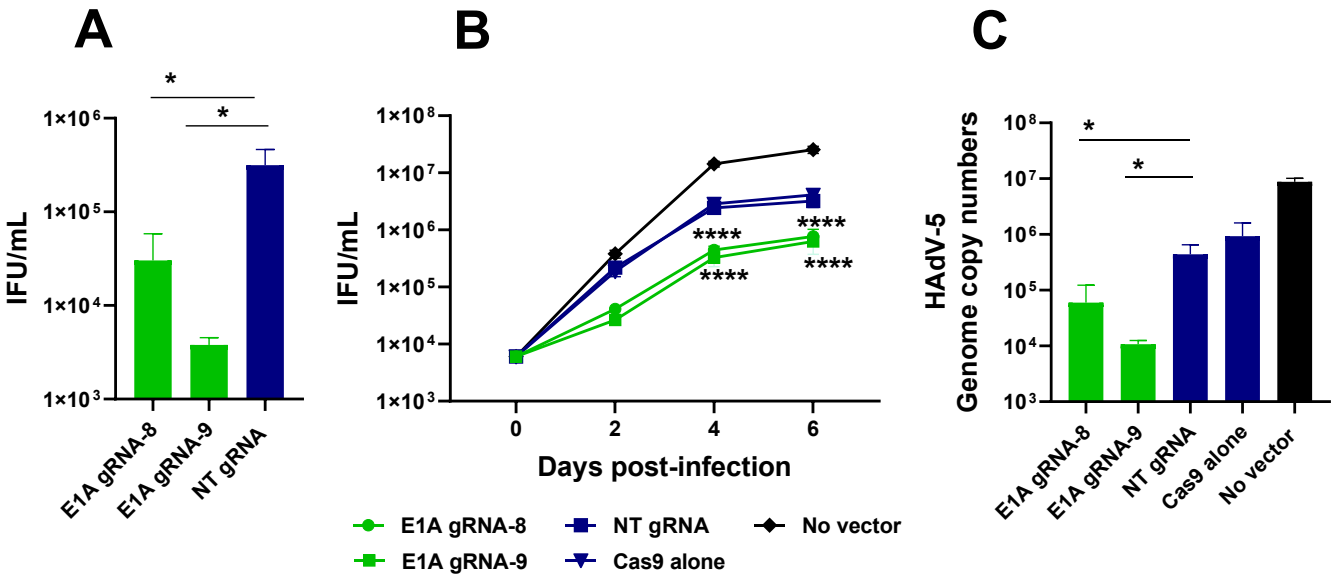

A
B
C
IFU/mL
1×10⁶
1×10⁵
1×10⁴
1×10³
*
*
E1A gRNA-8
E1A gRNA-9
NT gRNA
1×10⁸
1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
0
2
4
6
Days post-infection
****
****
****
****
E1A gRNA-8
E1A gRNA-9
NT gRNA
Cas9 alone
No vector
HAdV-5
Genome copy numbers
10⁸
10⁷
10⁶
10⁵
10⁴
10³
*
*
E1A gRNA-8
E1A gRNA-9
NT gRNA
Cas9 alone
No vector

Figure 5

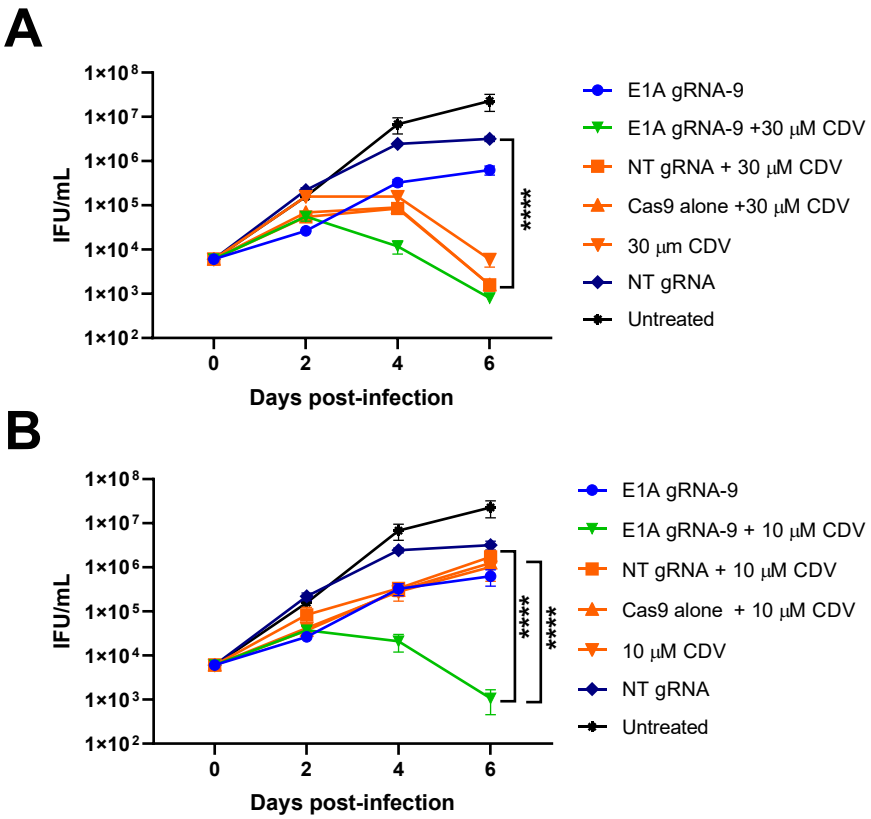

A
1×10⁸
1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
IFU/mL
0
2
4
6
Days post-infection
****
E1A gRNA-9
E1A gRNA-9 +30 μM CDV
NT gRNA + 30 μM CDV
Cas9 alone +30 μM CDV
30 μm CDV
NT gRNA
Untreated
B
1×10⁸
1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
IFU/mL
0
2
4
6
Days post-infection
****
****
E1A gRNA-9
E1A gRNA-9 + 10 μM CDV
NT gRNA + 10 μM CDV
Cas9 alone + 10 μM CDV
10 μM CDV
NT gRNA
Untreated

Figure 6

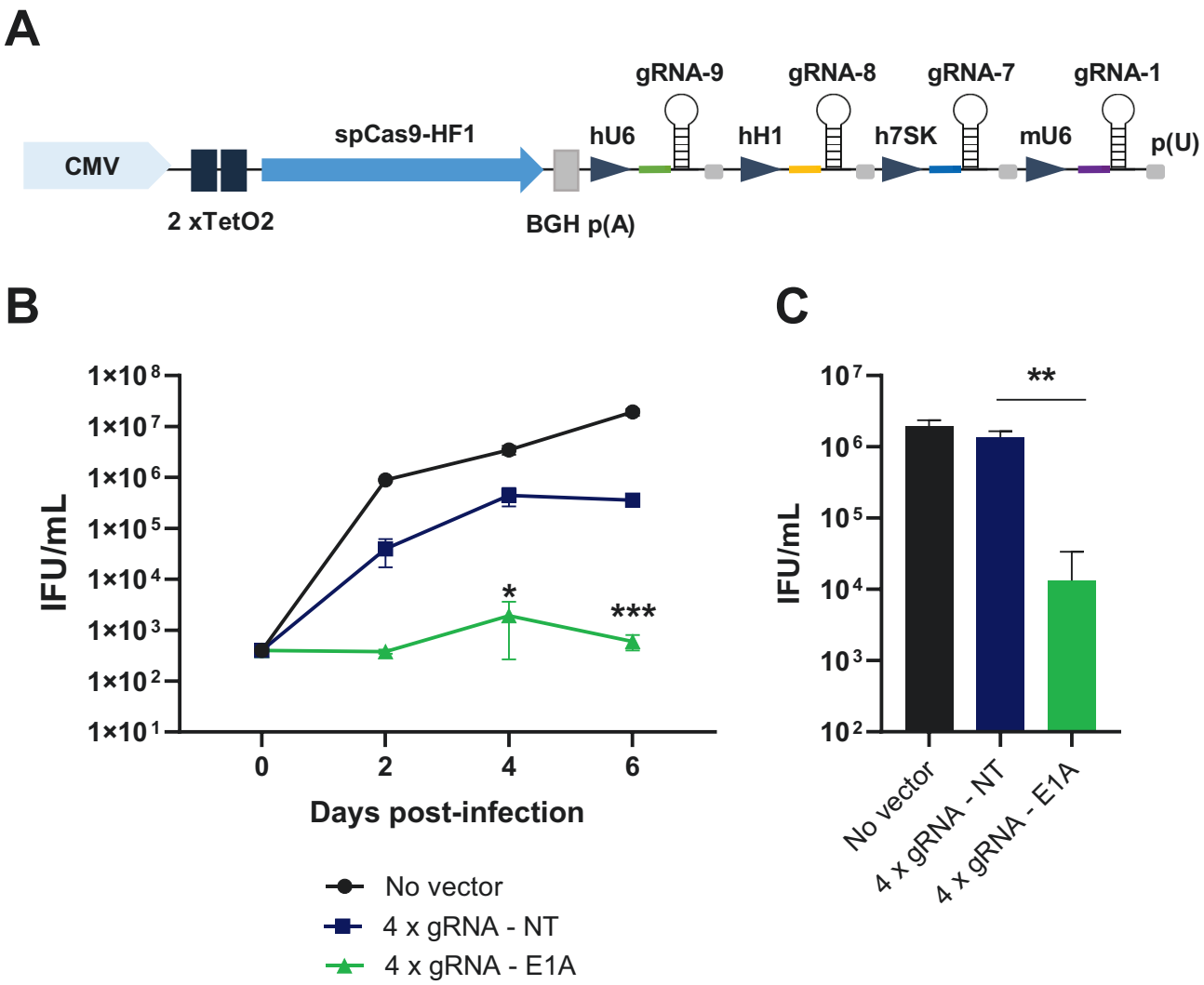
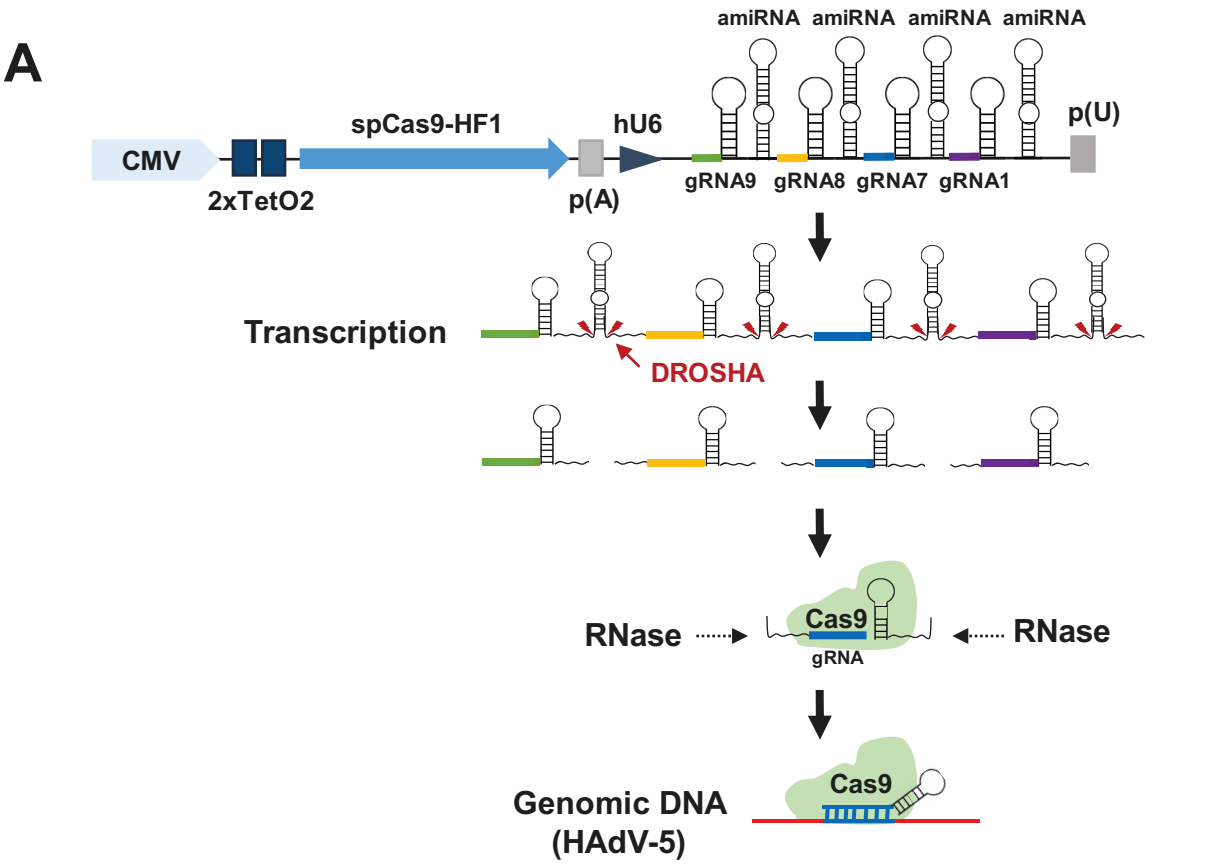
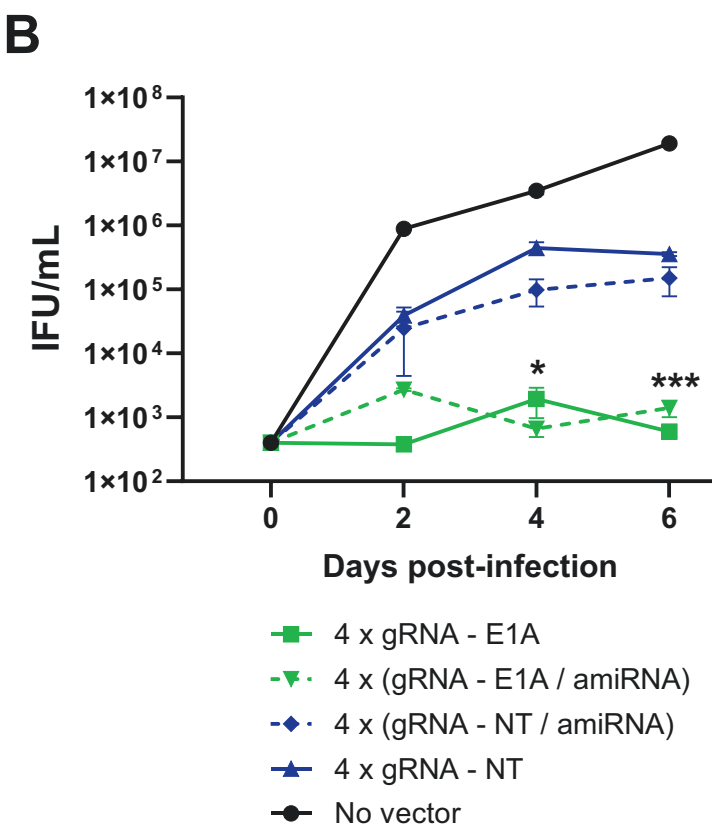

A
gRNA-9
gRNA-8
gRNA-7
gRNA-1
spCas9-HF1
hU6
hH1
h7SK
mU6
p(U)
CMV
2 xTetO2
BGH p(A)
B
IFU/mL
1×10 8
1×10 7
1×10 6
1×10 5
1×10 4
1×10 3
1×10 2
1×10 1
0
2
4
6
Days post-infection
*
***
No vector
4 x gRNA - NT
4 x gRNA - E1A
C
IFU/mL
10 7
10 6
10 5
10 4
10 3
10 2
**
No vector
4 x gRNA - NT
4 x gRNA - E1A

Figure 7


A
amiRNA amiRNA amiRNA amiRNA
CMV
2xTetO2
spCas9-HF1
p(A)
hU6
gRNA9 gRNA8 gRNA7 gRNA1
p(U)
Transcription
DROSHA
RNase
Cas9
gRNA
RNase
Genomic DNA
(HAdV-5)
Cas9


B
IFU/mL
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
1×10^2
0
2
4
6
Days post-infection
*
***
4 x gRNA - E1A
4 x (gRNA - E1A / amiRNA)
4 x (gRNA - NT / amiRNA)
4 x gRNA - NT
No vector

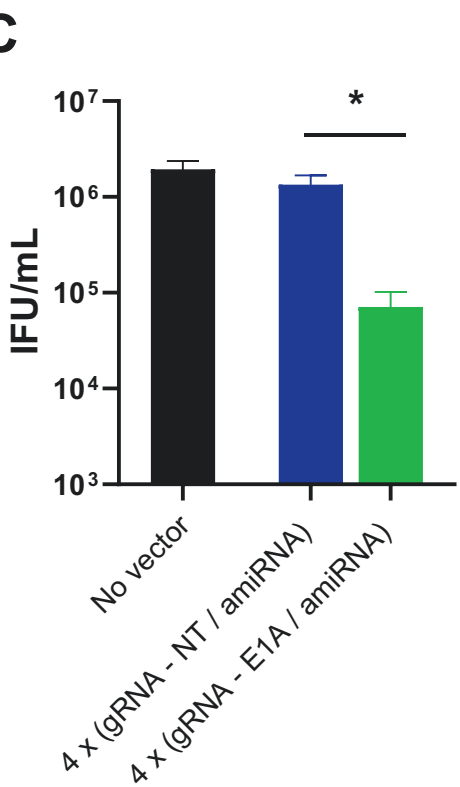

C
IFU/mL
10^7
10^6
10^5
10^4
10^3
*
No vector
4 x (gRNA - NT / amiRNA)
4 x (gRNA - E1A / amiRNA)

Figure 8

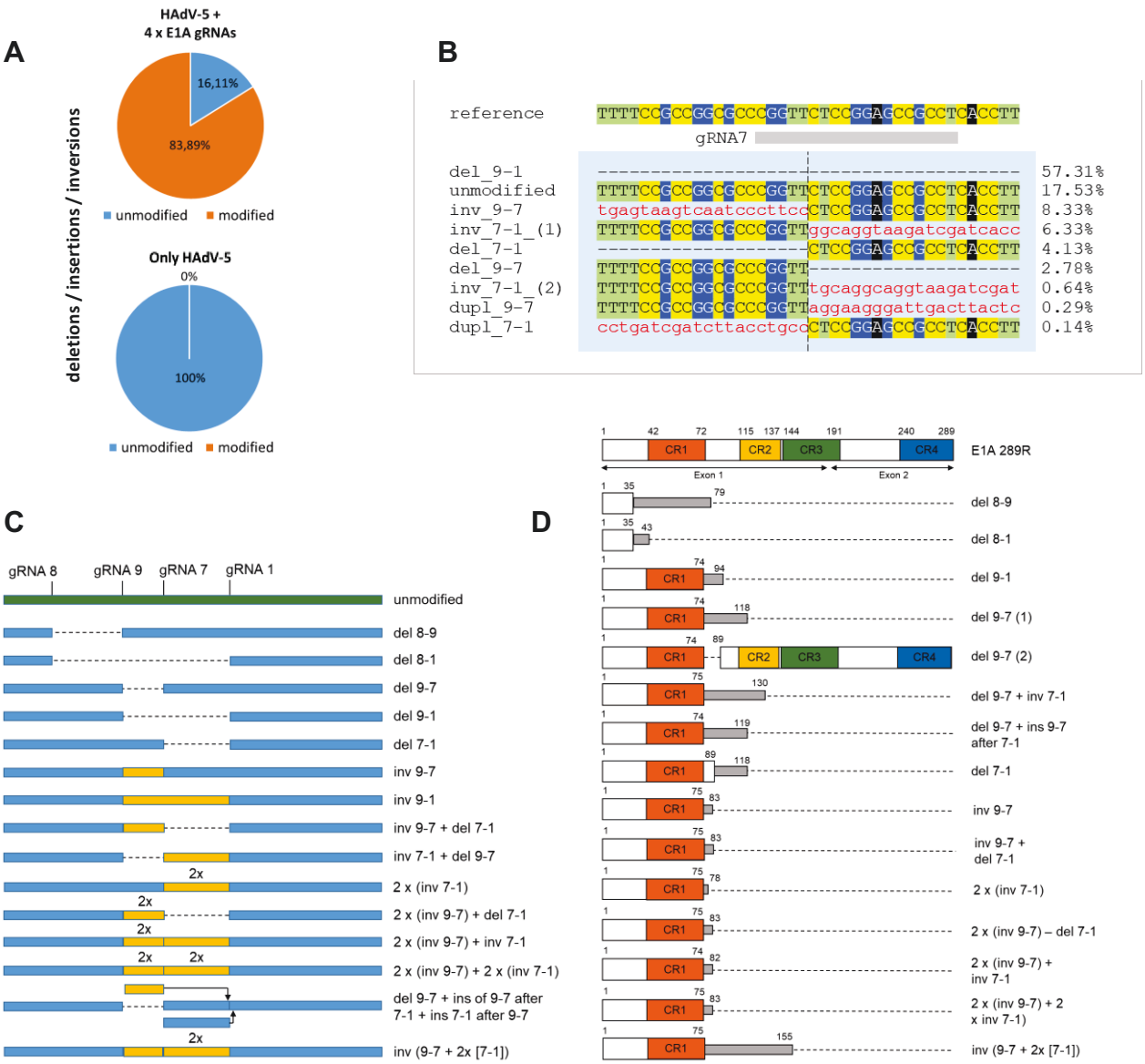

A
HAdV-5 +
4 x E1A gRNAs
16,11%
83,89%
unmodified
modified
deletions / insertions / inversions
Only HAdV-5
0%
100%
unmodified
modified
B
reference
TTTTCCGCCGGCGCCCGGTTCTCCGGAGCCGCCTCACCTT
gRNA7
del_9-1 57.31%
unmodified 17.53%
inv_9-7 8.33%
inv_7-1_(1) 6.33%
del_7-1 4.13%
del_9-7 2.78%
inv_7-1_(2) 0.64%
dupl_9-7 0.29%
dupl_7-1 0.14%
C
gRNA 8
gRNA 9
gRNA 7
gRNA 1
unmodified
del 8-9
del 8-1
del 9-7
del 9-1
del 7-1
inv 9-7
inv 9-1
inv 9-7 + del 7-1
inv 7-1 + del 9-7
2 x (inv 7-1)
2 x (inv 9-7) + del 7-1
2 x (inv 9-7) + inv 7-1
2 x (inv 9-7) + 2 x (inv 7-1)
del 9-7 + ins of 9-7 after 7-1 + ins 7-1 after 9-7
inv (9-7 + 2x [7-1])
D
E1A 289R
Exon 1
Exon 2
CR1
CR2
CR3
CR4
del 8-9
del 8-1
del 9-1
del 9-7 (1)
del 9-7 (2)
del 9-7 + inv 7-1
del 9-7 + ins 9-7 after 7-1
del 7-1
inv 9-7
inv 9-7 + del 7-1
2 x (inv 7-1)
2 x (inv 9-7) – del 7-1
2 x (inv 9-7) + inv 7-1
2 x (inv 9-7) + 2 x inv 7-1)
inv (9-7 + 2x [7-1])

Table S1: Primer List

Primer names	**5´-3´**
pENTR4 FW	GGAAAGAACCGGCGCGCCAAGCTTGAATTCGCGGCCGCACTC
pENTR4 RV	AAGCAGATTCGACTGAATTGGTTCCCATGGTG
CMV Tet02_FW	CAATTCAGTCGAATCTGCTTAGGGTTAG
CMV Tet02_FW	ATAGTGAGTCGTTTAAACGCTAGAGTCC
Cas9 HF1 FW	GCGTTTAAACGACTCACTATAGGGAGAGCC
Cas9 HF1 RV	TTGGCGCGCCGGTTCTTTCCGCCTCAGAAG
RV01 sequencing FW	GTTCCAGTACGGCTCCAAG
RV01 sequencing RV	CTGGCGGCCGCTTTACTTG
T7E1 E1A Set1 FW	CAGCGAGTAGAGTTTTCTCC
T7E1 E1A Set1 RW	GTAGACAAACATGCCACAGG
T7E1 E1A Set2 FW	CGGTGAGTTCCTCAAGAG
T7E1 E1A Set2 RW	CCAAACCCACCACTCTATC
T7E1 E1A Set3 FW	GTCAGCTGACGTGTAGTG
T7E1 E1A Set3 RW	CCGTACTACTATTGCATTCTCTAG
E3 qPCR FW	TGCTGCACTGCTATGCTAAT
E3 qPCR RV	TCCTCAATAAAGCTGCGTCTG
E3 qPCR probe	TGCTCGCTTTGGTCTGTACCCTAC
E1A/E1B loop seq FW	TACCCGGTGAGTTCCTCAAG
E1A/E1B loop seq RV	GCACCCATCCCAGCTTAACC
Cas9 qPCR FW	ACGGGATAAGAGACAAGCAAAG
Cas9 qPCR RV	GGTTAAAGAGTCATCATGGATCAG
Cas9HFqPCR_probe	TAAAGAGCGACGGCTTCGCCAATA
OTUD5 F1	ATGAGAGAGAGTGCAGGGGT
OTUD5 R1	TCACAGGCCTAGCAGATCCT
IFFO1 F1	GCTCCCACCTCCCTTCAAAA
IFFO1 R1	GTGGGTGTCTGCTACTTCCC
GPR85 F1	GTGTGCTCAGTCCAAGAGGG
GPR85 R1	TGAGGAGTCAAGAGCAACGG
UNC80 F2	GCCATTTTCAGAGGAGGACA
UNC80 R2	ACGTATGCAAGAGGACACACC
RNF111 F1	CGTGGCACTATCACATTATTTACAG
RNF111 R1	GTCTTTCCACAGGGCAAGCA
FOXJ2 F2	GGAGAGGCCACATTACCAAG
FOXJ2 R2	TTGTCCTCATGCCCTACCAT
CTD-3060P21.1/RAP1GAP2 F1	CTGCCTGCTGCTGTCTTAGT
CTD-3060P21.1/RAP1GAP2 R1	TCCGGAATCTGCCCTCAGTA

TOB1-AS1 F1	GTCTGGAAAGGGACTGTGGG
TOB1-AS1 R1	TAAACGGATCCGAGTCGCAG
ABCF3 F1	CCTGAGGAGGAGTACCGTCA
ABCF3 R1	CTGAAAGACAGGAGGGCAGG
TMEM110 F1	CACCACCACCTGGATCTCAC
TMEM110 R1	TGAGAGCAGATCAGAGGGCT
PRKACB F1	CCACCATGGTGTTCTGGAGG
PRKACB R1	TGTAGCATCAAAAGAAGGCCA
RP5-964N17.1 F1	TGCTGTCTAAGTTCTGCGCC
RP5-964N17.1 R1	GCCCAGTCAGCTACAGAGTG

Table S2: Surrogate Reporter Vector Target Sequences

Vector name	Target sequence inserted into RV01	5´-3´
RVO1-1,3,4	E1A gRNA 1,3,4	GAATTCAACCTTGTACCGGAGGTGATCGATCTTACCTG CCACGAGGCTGGCTGGATCC
RVO1-2,9	E1A gRNA 2,9	GAATTCCCCCCGAAGATCCCAACGAGGAGGCGTGTAA TGTTGGCGGTGCAGGAAGGGAGGATCC
RVO1-5,6,8	E1A gRNA 5,6,8	GAATTCGCCAGTCTTTTGGACCAGCTGATCGTCCACCT CCTAGCCATTTTGAACCACCTACGGATCC
RVO1-7,10	E1A gRNA 7,10	GAATTCGCCCGGTTCTCCGGAGCCGCCTCACCTTTCC CGGCAGCCCGAGCAGCCGGGATCC

Figure S1

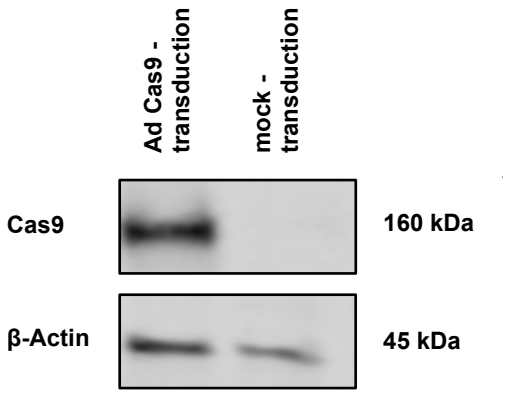


Figure S1. Expression of spCas9-HF1.

The functionality of the spHF-Cas9 expression cassettes harboring the doxycycline-regulatable CMV promoter which is present in all adenoviral CRISPR/Cas9 vectors was proven in the absence of doxycycline in HeLa cells lacking the tetracycline repressor. Equal amounts of protein from HeLa cells at 48 h post-transduction were subjected to Western blot analysis for the detection of spHF-Cas9 and for comparison of □-actin. The expression is exemplarily shown for the vector expressing only spCas9-HF1.

Figure S2

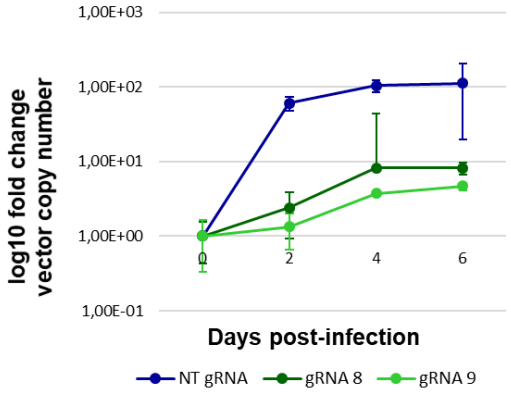


Figure S2. Replication of the adenoviral vectors in HAdV-5-infected cells.

HeLa cells were transduced with the adenoviral vectors containing Cas9 in combination with the targeting gRNAs 8 or 9 or with a non-targeting (NT) gRNA at an MOI of 30. 24 h after transduction the cells were infected with HAdV-5 at an MOI of 0.1. Vector copy numbers at time points 0, 2, 4, and 6 days post-infection were determined by qPCR with primers/probe specific for the Cas9-encoding part of the vectors. Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment.

Figure S3

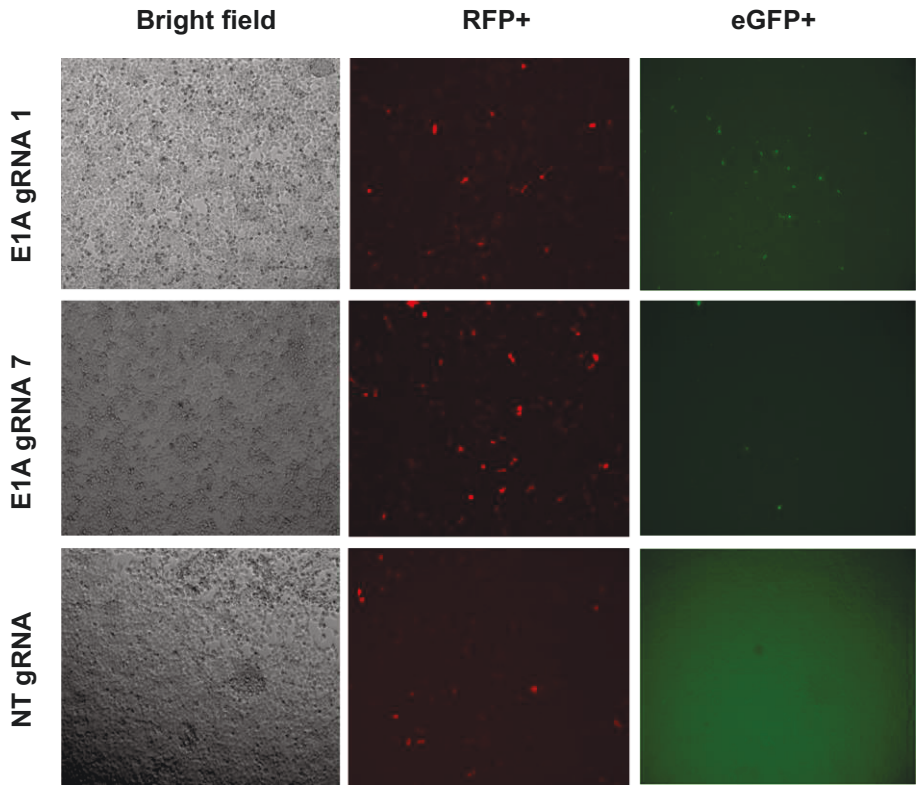


Figure S3. Detection of gene editing by E1A-targeting gRNAs 1 and 7 in surrogate reporter assays.

HEK293 cells were transfected with the surrogate reporter vector and transduced with the recombinant adenoviral vectors expressing either a targeting or a non-targeting (NT) gRNA. Fluorescence was monitored 48h post-transduction with a Leica DMi8 System. Bright field, red and green fluorescence images at a magnification of 10x are shown. Microscopy settings were: HC PL FLUOTAR CS 10x/0.40 DRY; Camera Leica DFC 360FX: active resolution 1392 x 1040, pixel bitdepth 12/8 bit, pixel size 6.45 µm x 6.45 µm; live image with 1392 x 1040 at 20 images/second.

Figure S4

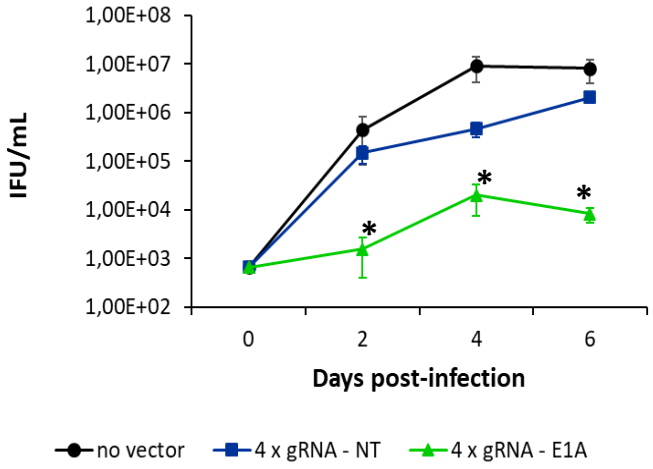


Figure S4. Inhibition of HAdV-5 replication in A549 cells.

A549 cells were transduced with the adenoviral vector containing Cas9 in combination with gRNAs 1, 7, 8, and 9 expressed from individual promoters or with a control vector carrying four non-targeting (NT) gRNAs at an MOI of 100 followed by infection of the cells with HAdV-5 at an MOI of 0.01 24 h later. Numbers of infectious virus particles were determined at the indicated time points and were expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of three infections. *$p < 0.05$; *** $p < 0.001$.

Figure S5

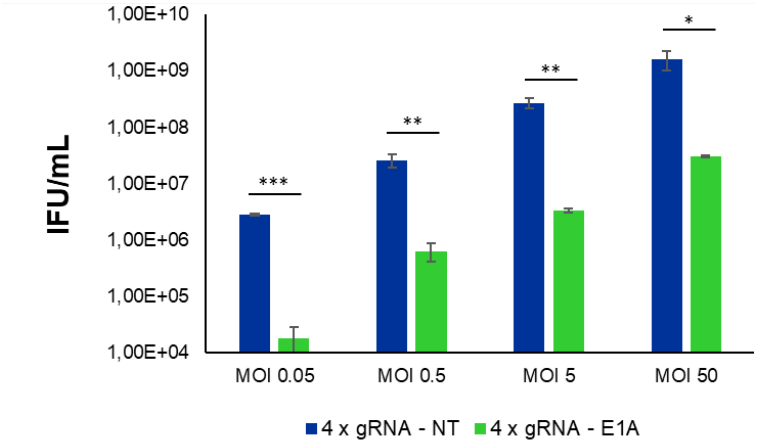


Figure S5. Inhibition of HAdV-5 replication at increased MOIs.

A549 cells were transduced with the adenoviral vectors containing Cas9 in combination with the E1A-targeting gRNAs 1, 7, 8, and 9 expressed from individual promoters or with a corresponding control vector carrying four non-targeting (NT) gRNAs instead of the targeting gRNAs at a constant MOI of 50. 24 h after transduction the cells were infected with HAdV-5 at increasing MOIs ranging from 0.05 to 50. 48 h after infection numbers of infectious virus particles were determined and were expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of triplicate infections of a representative experiment. *p < 0.05; ** p < 0.01; *** p < 0.001.

Figure S6

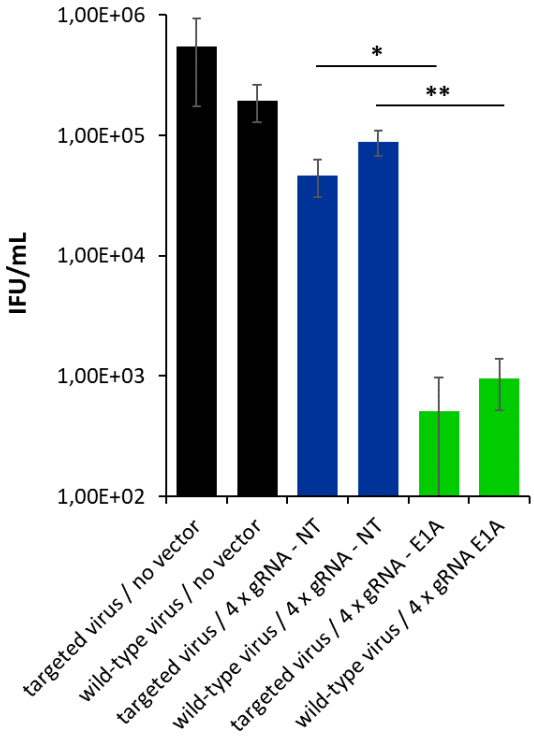


Figure S6. CRISPR/Cas9-mediated inhibition of replication of virus recovered from the first round of targeting by CRISPR/Cas9

HeLa cells were transduced with the adenoviral vector containing Cas9 in combination with gRNAs 1, 7, 8, and 9 expressed from individual promoters (green), with a control vector carrying four non-targeting (NT) gRNAs (blue), or were mock-transduced (black) at an MOI of 100. 24 h later cells were infected with virus recovered after the first round of targeting by CRISPR/Cas9 (day 6 time point; virus pooled from three independent experiments) or with non-targeted wild-type virus at an MOI of 0.01. Virus was allowed to replicate for two days. At day 2 post-infection numbers of infectious virus particles were determined and were expressed as IFU/mL (infectious units per mL). Data represent the means (n = 3) ± SD of three infections. *$p < 0.05$, **$p < 0.01$.

Figure S7

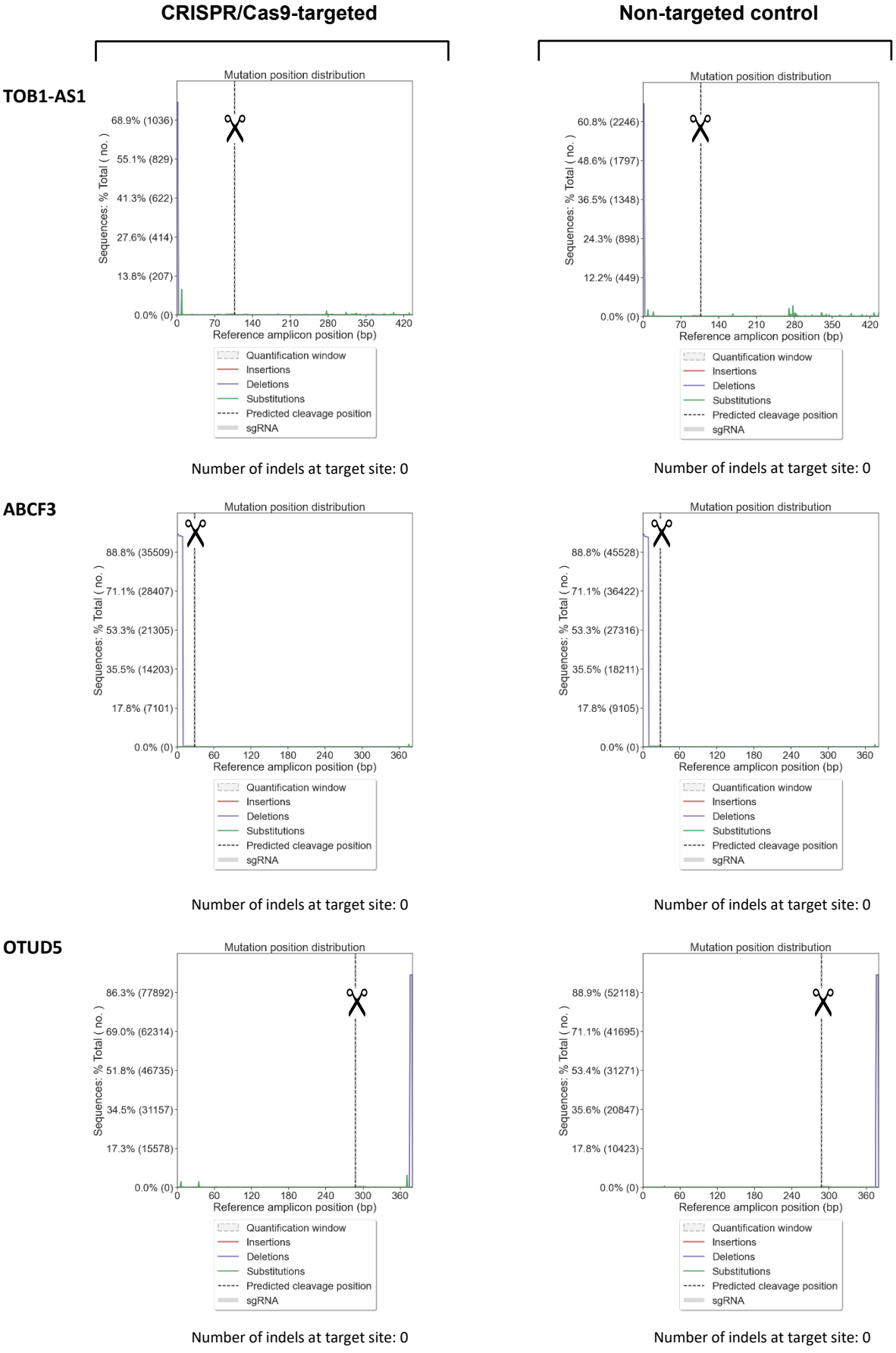
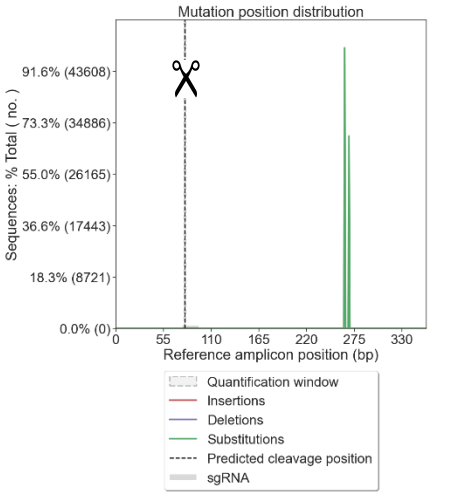

CRISPR/Cas9-targeted
Non-targeted control
TOB1-AS1
Mutation position distribution
Sequences: % Total (no.)
68.9% (1036)
55.1% (829)
41.3% (622)
27.6% (414)
13.8% (207)
0.0% (0)
0 70 140 210 280 350 420
Reference amplicon position (bp)
Quantification window
Insertions
Deletions
Substitutions
Predicted cleavage position
sgRNA
Number of indels at target site: 0
Mutation position distribution
Sequences: % Total (no.)
60.8% (2246)
48.6% (1797)
36.5% (1348)
24.3% (898)
12.2% (449)
0.0% (0)
0 70 140 210 280 350 420
Reference amplicon position (bp)
Quantification window
Insertions
Deletions
Substitutions
Predicted cleavage position
sgRNA
Number of indels at target site: 0
ABCF3
Mutation position distribution
Sequences: % Total (no.)
88.8% (35509)
71.1% (28407)
53.3% (21305)
35.5% (14203)
17.8% (7101)
0.0% (0)
0 60 120 180 240 300 360
Reference amplicon position (bp)
Quantification window
Insertions
Deletions
Substitutions
Predicted cleavage position
sgRNA
Number of indels at target site: 0
Mutation position distribution
Sequences: % Total (no.)
88.8% (45528)
71.1% (36422)
53.3% (27316)
35.5% (18211)
17.8% (9105)
0.0% (0)
0 60 120 180 240 300 360
Reference amplicon position (bp)
Quantification window
Insertions
Deletions
Substitutions
Predicted cleavage position
sgRNA
Number of indels at target site: 0
OTUD5
Mutation position distribution
Sequences: % Total (no.)
86.3% (77892)
69.0% (62314)
51.8% (46735)
34.5% (31157)
17.3% (15578)
0.0% (0)
0 60 120 180 240 300 360
Reference amplicon position (bp)
Quantification window
Insertions
Deletions
Substitutions
Predicted cleavage position
sgRNA
Number of indels at target site: 0
Mutation position distribution
Sequences: % Total (no.)
88.9% (52118)
71.1% (41695)
53.4% (31271)
35.6% (20847)
17.8% (10423)
0.0% (0)
0 60 120 180 240 300 360
Reference amplicon position (bp)
Quantification window
Insertions
Deletions
Substitutions
Predicted cleavage position
sgRNA
Number of indels at target site: 0

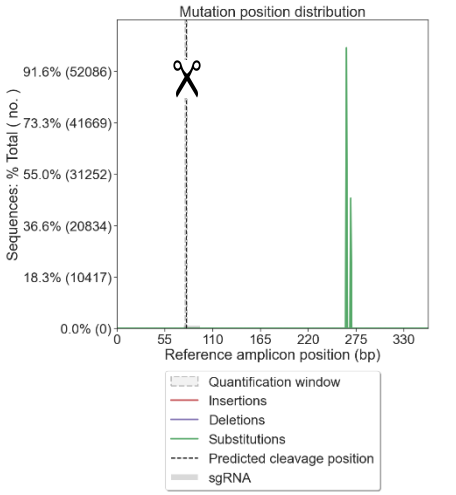
Figure S7 - continued

CRISPR/Cas9-targeted | **Non-targeted control**

IFFO1

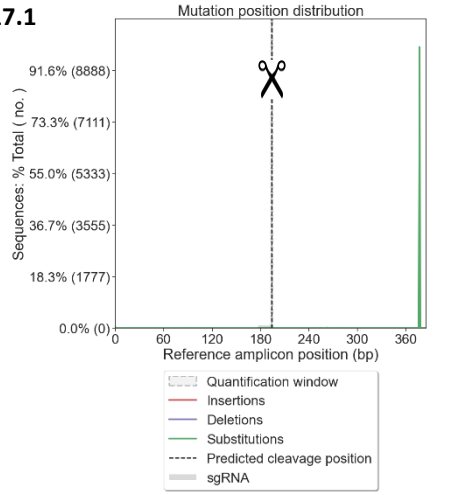


Number of indels at target site: 0

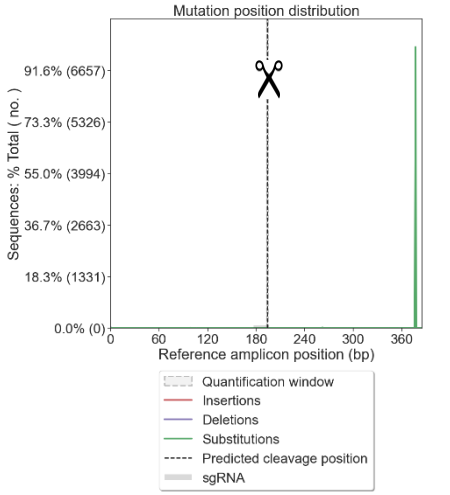


Number of indels at target site: 0

RP5-964N17.1

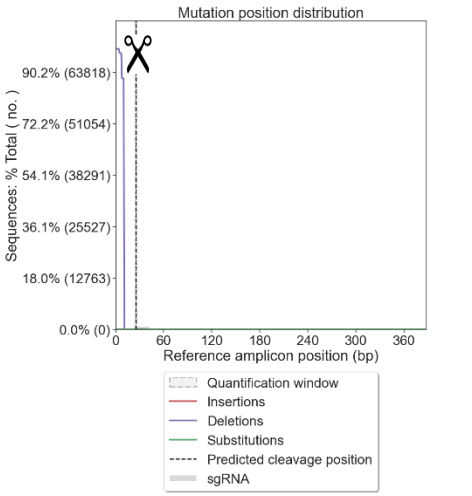


Number of indels at target site: 0

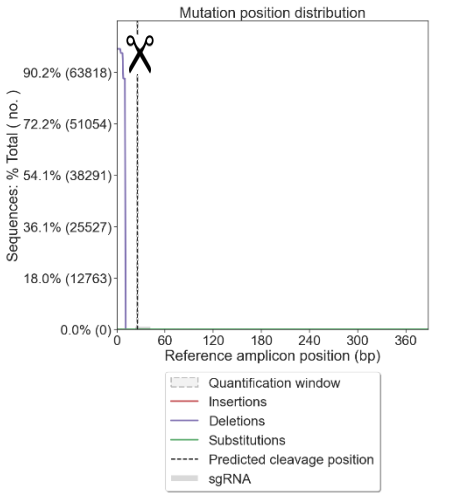


Number of indels at target site: 0

GPR85



Number of indels at target site: 0



Number of indels at target site: 0

Figure S7 - continued

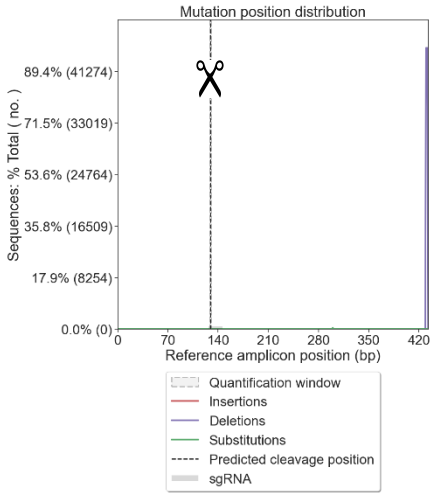
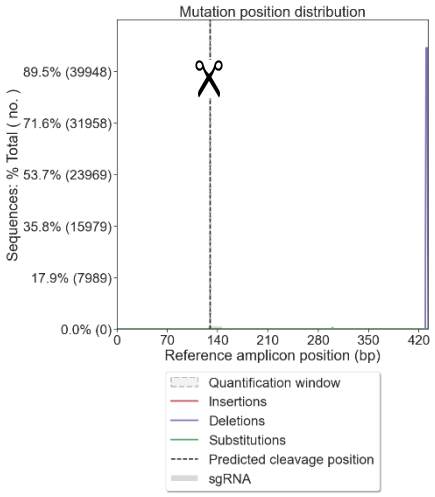
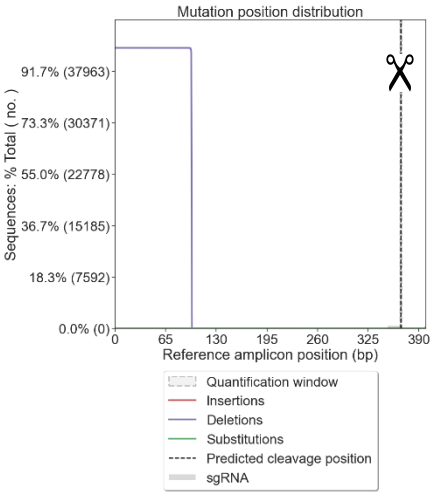
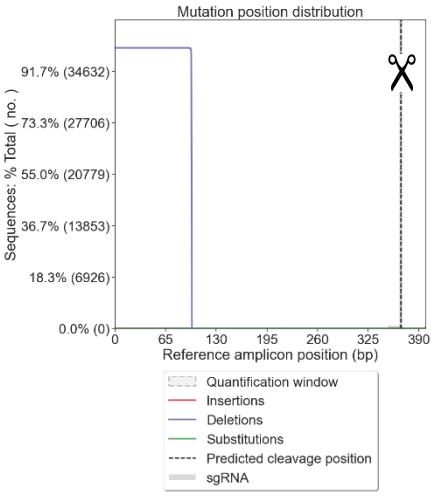
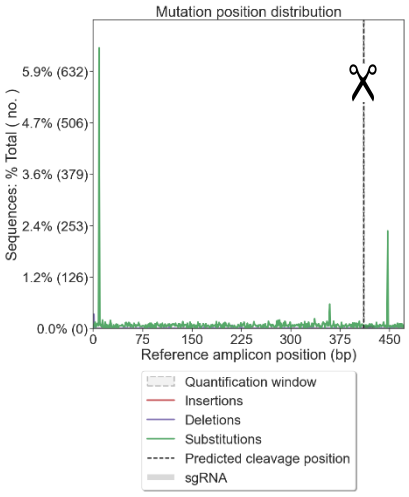
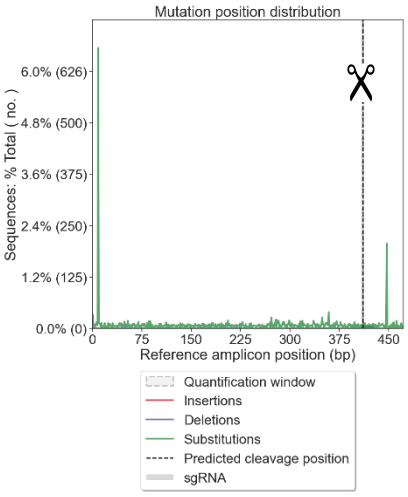
	CRISPR/Cas9-targeted	Non-targeted control
PRKACB		
	Number of indels at target site: 0	Number of indels at target site: 0
TMEM110		
	Number of indels at target site: 0	Number of indels at target site: 0
UNC80		
	Number of indels at target site: 0	Number of indels at target site: 0

Figure S7 - continued

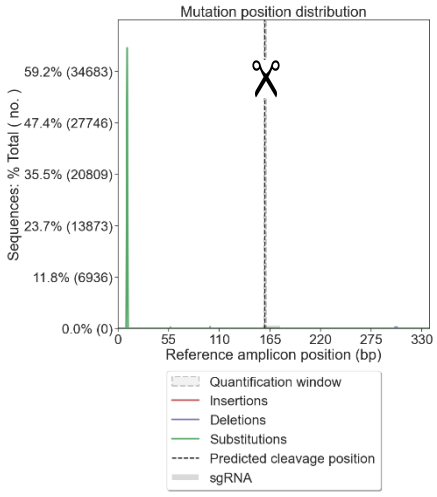
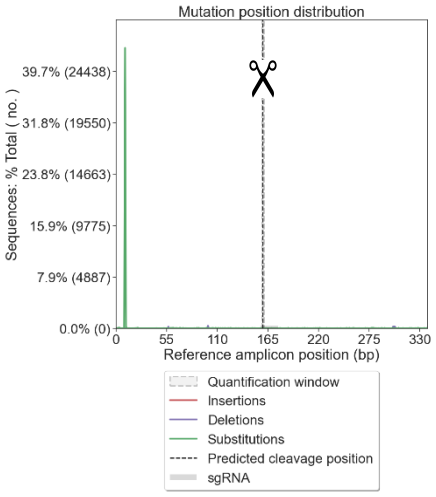
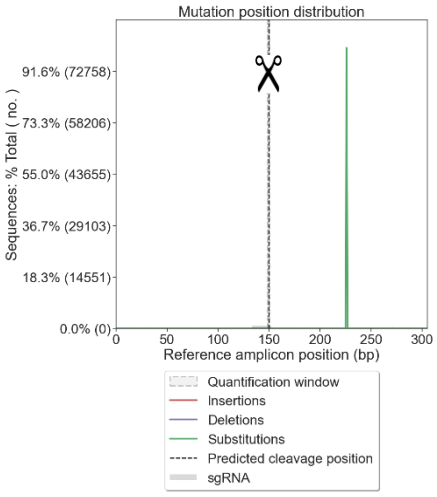
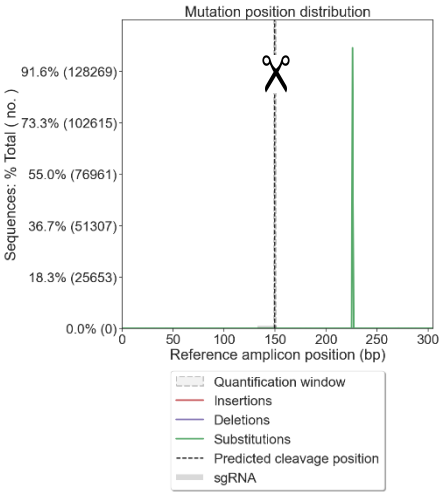
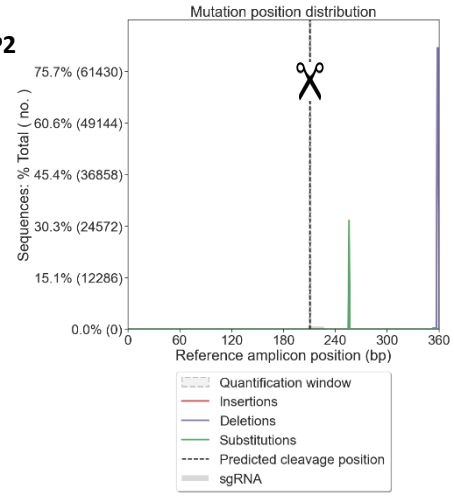
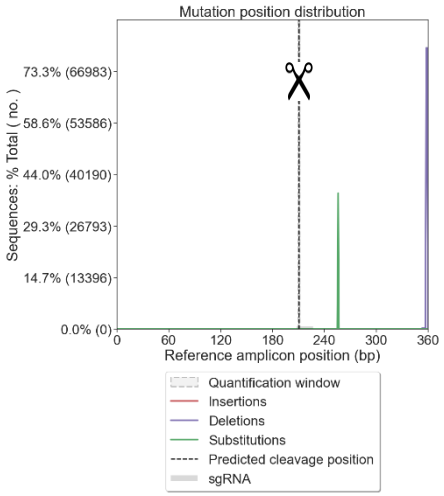
	CRISPR/Cas9-targeted	Non-targeted control
RNF111		
	Number of indels at target site: 0	Number of indels at target site: 0
FOXJ2		
	Number of indels at target site: 0	Number of indels at target site: 0
CTD-3060P21.1/ RAP1GAP2		
	Number of indels at target site: 0	Number of indels at target site: 0

Figure S7. CRISPR/Cas9-mediated cleavage at potential off-target sites.

HeLa cells were transduced with the adenoviral vectors harboring Cas9 in combination with the four gRNAs 1, 7, 8 and 9 expressed from separate promoters at an MOI of 100 or were mock-transduced. 24 h after transduction the cells were infected with HAdV-5 at an MOI of 0.01. Two days post-infection DNA was isolated from the cells, the DNA regions comprising the potential off-target sites were amplified by PCR, the amplicons were subjected to next generation sequencing, and the sequencing reads were mapped to the respective reference amplicon sequences. Percentages of modifications around the indicated potential off-target sites in cells targeted with CRISPR/Cas9 (left) and in non-targeted control cells (right) representing background noise as a result of randomly occurring PCR amplification/sequencing were calculated with CRISPResso2. Insertions, deletions, and substitutions are indicated. Deletions (blue) appearing close to the borders of the graphs do not represent CRISPR/Cas9-mediated modifications but indicate the ends of the amplicons that were not covered by sequencing. Substitutions (green) occurring at more or less the same frequencies at the same positions in targeted and non-targeted cells represent mutations that have accumulated in the HeLa cell population that was used for the experiments. The potential cleavage sites at which modifications would appear in case of off-targeting are indicated with dashed lines and pairs of scissors.

4 Conclusion and Outlook

Human pathogenic viruses remain a persistent challenge to the community, both locally and globally (Luo & Gao, 2020). Human adenoviruses are a notable example. They are non-enveloped small double-stranded DNA respiratory pathogens (Ismail et al., 2018). In the majority of the population, adenovirus infections are quickly cleared up by a competent immune system, with only temporary flu-like symptoms present. This usually leads to life-long immunity of the host. However, in immunocompromised patients, infections are a major risk and high mortality rates observed (Lynch et al., 2011). While treatment advancements were made using off-label medication (Londeree et al., 2020) and new ways are explored (Ibrisimovic et al., 2013; Kneidinger et al., 2012), overall, the situation still remains unsatisfactory. Currently, there are no approved antiviral drugs, targeting adenovirus infection, available (CDC, retrieved 2023).

In this thesis I present the development of a step-by-step optimized CRISPR/CasRx and CRISPR/Cas9 antiviral system to target adenoviral infections. The construct's inhibitory efficacy was evaluated in wild-type adenovirus replication assays.

In the first topic I evaluated the antiviral properties of CRISPR/CasRx. Reduction rates of virus infectious units between 1,5 and 2 orders of magnitude were achieved across multiple days post wild-type infection and across varying assay designs. This translates into a repression by up to 99,66% underlining the promising potential CasRx may have. A potential further reinforced by the fact that a highly replicative DNA virus was successfully targeted via it's RNA stage. Delivery of the systems components to cells by adenovirus based recombinant viral particles was straightforward and efficient, however, possible self-targeting capabilities needed to be managed closely. Future projects may take the expression cassette of the optimized best performing candidate to transfer it into a more advanced and less immunogenic viral delivery vector than the one employed in this study. Subsequent *in vivo* studies may shed additional light on the real-world treatment potential of this proposed antiviral platform and thus might be another step closer to the clinic.

In the second topic I combined CRISPR/CasRx with RNA interference, mediated through artificial micro RNAs, to improve antiviral efficacy possibly further. I generated multiple vectors, all following different approaches for CasRx/amiRNA multiplexing, and subsequently tested them in reporter assays to identify the best design. I found out that expression of amiRNAs under the CasRx promoter with added mRNA stabilizing structures and expression under the gRNA promoter using processed gRNA design

were the best approaches. Future projects may build up on that knowledge by generating recombinant viral vectors using the described designs. Additionally, the constructs may benefit further from the simultaneous expressing of multiple gRNAs and multiple amiRNAs. Testing them in wild-type inhibition assays, shall give answers on their efficacy and how they compare to single guide CasRx vectors.

In the third topic I presented the development of a CRSIPR/Cas9 based antiviral platform and its evaluation in the context of wild-type adenovirus infection. This DNA targeting system showed a reduction of virus infectious units up to 1,9 orders of magnitude (98,8%) when targeting the coding regions of early expressed viral gene *E1A*, essential for replication. Application in combination with an additional antiviral agent, cidofovir, further improved the inhibition to 3,47 orders of magnitude (99,97%). CRIPSR/Cas9 mediated double-strand breaks in one DNA locus only, may lead to the emergence of escape mutants, resistant progeny that would likely outperform the antiviral platform. As counter strategy, we decided to generate four-gRNA vectors, targeting the viral genome in multiple different locations, thus greatly reducing the likelihood of viral escape (Darcis et al., 2019). Two ways of multiplexing were explored. One having each guide under its own promoter, showing reduction rates of up to 2,8 orders of magnitude (99,8%), and the other having four guides split up by amiRNAs under the same promoter, leading to a significantly reduced size of the expression cassette, showing inhibition by up to three orders of magnitude. Subsequent next generation sequencing revealed detailed insights into the targeting process. 83,89% of the total reads showed mutations, with deletions, found between all targeting sites, the most prominent outcome. Almost all of them lead to frameshifts, and consequently truncated E1A proteins, explaining the high inhibitory efficacy, observed in wild-type replication assays. The results indicate a promising antiviral performance of the proposed platform in targeting adenovirus replication and that CRISPR/Cas9 is able to cope with the rapidly increasing viral DNA amount during the infection cycle. Similar to topic one, the next steps should include moving to a more advanced delivery system and *in vivo* studies.

To conclude, the results indicate that the proposed antiviral treatment platform successfully targeted the adenoviral replication cycle and that it may be a promising candidate for further research, with the aim of treating patients in the future and thus adding to the limited therapy repartiour, available today. I believe this thesis will contribute to the knowledge of possible ways to treat adenoviral- and viral infections in general and will add to the understanding of antiviral CRISPR/Cas applications.

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