nature portfolio

Peer Review File

ALCAM is an Entry Factor for Severe Community Acquired Pneumonia-associated Human Adenovirus Species B

Corresponding Author: Professor Jia Liu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Xie et al. describe the identification of ALCAM as entry receptor of several species B adenoviruses including pneumonia-associated types. The authors initially found that besides influenza, adenovirus was the second most frequently identified virus infection after analyzing pathogens during severe community-acquired pneumonia (SCAP). They subsequently identified HAdV-B7 as the pathogen which was most frequently detected besides HAdV-B3 and -B55. Using a CRISPR-based library screen to perform a broad knockout of surface receptors, the authors identified ALCAM as entry receptor besides DSG-2. Next Xie and colleagues explored a chimeric HAdV-C5 vector carrying a B7 fiber and they produced ALCAM knockout cells to analyze entry of the GFP encoding chimeric vector. The authors found that DSG-2 influences virus attachment and entry and that ALCAM is solely involved in entry. After generating different variants of fiber and ALCAM encoding plasmids, the authors performed co-IP analyses and identified the fiber domain interacting with ALCAM. Furthermore, the authors show evidence that HAdV-B7 entry depends on the EndoA3 axis. In addition, they performed SPR analyses of ALCAM and HAdV-B7 fiber knob domains and competitions assays to further investigate binding characteristics. The evidence supporting this claim is overall solid, statistics are rigorously calculated and the authors do not make excessive claims. This is a logical study but for publication further experiments and analyses need to be performed to strengthen the main conclusion. In total, publication of this article can be supported if the authors address the following comments.

Major comments:

• The authors conclude that ALCAM represents a general entry receptor for species B adenoviruses. However, this is not entirely true because Ad35 seems not to be involved and the authors mainly checked chimeric viruses and not wild type viruses. To strengthen this hypothesis the authors should perform experiments with wild type viruses, which can be ordered and/or obtained from reference laboratories.
• The Kd measured for knob binding to ALCAM should be discussed. Baker et al. (2019, Nature Communications) found a Kd of 66µM between HAdV-B3 knob and DSG-2, suggesting the 700nM value for ALCAM bears witness to a high affinity. However, since the data points do not fit the calculated curve well the actual Kd may be somewhat higher. If this is feasible for the authors, it would be valuable to complete the SPR measurements with HAdV-B7 fiber interactions with DSG-2, or between full virus particles and ALCAM, to verify that the use of purified isolated protein domains does not introduce biases. Furthermore, the authors should include a specificity control in the SPR assay, for an example anti-tag antibodies that should induce a second response peak when added after tagged knobs.
• In Figure 1h DSG-2 seems to be a much stronger receptor compared to ALCAM. Have the authors compared binding strength of the HAdV-B7 fiber protein to ALCAM and DSG-2? This could be analyzed by SPR analyses.
• Since ALCAM is not involved in attachment, it can be hypothesized to be a co-receptor of DSG-2 or binding factors present in the extracellular matrix. A valuable addition to the paper would be to identify this attachment factor. If this is DSG-2, this could be easily investigated by the authors based on available materials and following the design of the EndoA3 experiment, by knocking down ALCAM in the DSG-2 knockout cell line and measuring if the decrease in infectivity is additive or not.
• Why do the authors assume that DSG-2 expression levels should be increased in SCAP patients? Is there any evidence in the literature that DSG-2 expression levels are enhanced after infection with adenovirus utilizing DSG-2 as entry receptor? Please clarify this in the discussion.
• Extended data Fig. 7f: The ALCAM band in the Western blot analyses seems to be very weak. Did the authors check

ALCAM expression levels on different cell lines from different origin? Have the authors checked for cell lines which naturally display low or high expression levels of ALCAM? These cell lines could be also used to study cell entry and would strengthen the data set.
• Discussion section: Maybe I missed that information but in my opinion, the authors should go into more detail about what is known regarding ALCAM (e.g. expression pattern in vivo, function, which cells express ALCAM). Moreover, the authors should discuss potentially other factors involved in uptake as for instance co-receptor usage or other factors which may influence transduction efficiencies as well.

Minor comments:

• Introduction section, line 56: There are >100 adenovirus types. Please correct.
• The fact that HAdV-C5 infectivity is not decreased by ALCAM knockout, and even increased, is an important control and should be discussed. It shows that ALCAM effect on species B adenoviruses is directly related to the virus infection itself and not to indirect influences through changes in cell growth, morphology or viability; that the fiber protein is indeed responsible for these effects, and not the Ad5 backbone used in this study; and questions whether HAdV-B35 infection is truly independent of ALCAM or if the lack of significance stems from opposite effects of ALCAM deletion on virus entry versus indirect cell effects.
• In figure 2, the effect of ALCAM knockout seems much more limited than that of its knockdown. Does this suggest ALCAM influences much more viability/growth than infection? The magnitude of ALCAM's involvement in infection in physiologically-relevant settings should be discussed.
• Is it correct that the "ALCAM KO" cell line is derived from HEK-293T and "ALCAM-/-" from HEK-293A? Please make it more explicit, the presence of both cell lines may confuse readers.
• In figure 4, the signification of abbreviations on the left of the pictures (e.g. "WCL") should be explained.
• Figure 4f suggests that isolated ALCAM C domain binds to the shaft as well. Please discuss.
• Extended figure 9 suggests that DSG-2 binds preferentially to monomers, then to trimers, then to dimers; while ALCAM has apparently a preference for dimers and hardly binds to trimers. The authors should discuss if this may be explained by technical biases or is representative of physiological conditions. One may hypothesize that the scarcity of intact fiber trimer binding to ALCAM explains its lack of involvement in attachment, while it becomes involved once the fibers start dissociating at the entry step.
• Figure 2: Was the surface expression of ALCAM after knockdown checked by flow cytometry and how do the author explain the remaining transduction efficiencies of more than 20% (Fig. 2g)?
• Extended data figure 2: Did the authors check DSG-2 and CAR receptor expression on the cell surface by flow cytometry before and after knockout? How do the authors explain that there are still ~20% transduction efficiencies with HAdV-C5?
• Cell clones for knockout cells were selected and single clones were chosen. However, how where the single cell clones selected and how and which clones were chosen for further use? By expression levels of the receptor or just by genome analyses?

Spelling mistakes
• Line 64: Introduction: "the second most prevalent viral pathogen" ("most" is missing)
• Line 77: "cellular proteins may be involved in the infection"
• Line 190: "ALCAM knockout cells exhibited reduced rate of infection" ("the" to be removed)
• Line 223: "ALCAM KO cells were incubated", not "was"
• Results section: "receptor usage", not "receptor utility" (three occurrences)
• Line 312: The sentence should be corrected. I think there is a "with" missing.
• Line 365: Methods: with "Cells of more than 500-fold coverage of the library size", do the authors mean that the number of collected cells is 500 times higher than the number of sgRNAs in the libraries?
• Line 480: "with a pore size of 0.45 µm", not µM

Reviewer #2

(Remarks to the Author)
In "ALCAM is A Functional Entry Receptor for Severe Community Acquired Pneumonia-associated Human Adenovirus Species B" Xie and Mei et al perform a targeted CRISPR KO screen to identify surface proteins that are necessary for Human Adenovirus B (HAdV-B) entry. Authors demonstrate that ALCAM deficiency provides a modest deficiency in HAdV-B infection likely at the step of viral internalization. A direct interaction between ALCAM and the HAdV knob was mapped. While the topic is of interest, the data is far from convincing that ALCAM is an entry receptor. Rather the data points to a quite modest facilitation of viral internalization by ALCAM1. Overall, the mechanistic data lacks rigor. A point by point critique follows:

Major Point

1. The phenotypic magnitude isn't compelling for a receptor. For example, in many panels the difference between WT or non-targeted samples is only 10% (e.g. Figures 2H, 3A, and throughout). This isn't consistent with other reports of HAdV receptors (e.g. PMID: 21151137). Furthermore, the presentation of the data with graphs starting at 85 or 90 percent of a normalized value casts doubt as to the role of this molecule in HAdV infection.

2. The authors conflate "entry" and "internalization". In figure 4, the authors are actually measuring viral internalization. An entry receptor would be considered to help the virus uncoat, fuse, or escape the endosome. The data presented suggests a modest decrease in internalization rather than these steps.

3. Throughout the manuscript the authors use a t-test when comparing more than two groups. A statistical test that accounts for multiple comparisons (e.g. ANOVA) is absolutely necessary especially given the modest magnitude.
4. Connections between ALCAM expression and SCAP are tenuous and don't provide insight into the biology. It is unclear why SCAP patients would have different expression of a HAdV receptor. Overall these connections detract from the main story.
5. Figure 4D-4F, many of the deletion mutants still interact. Thus it is imperative to include a negative control protein for the interactions studies (e.g. MBP or GFP or even an HAdV-C5 fiber). It is quite likely that these are sticky proteins that have non-specific interactions via Co-IP

Minor Points
1. The writing could be improved in many areas.
2. The authors are commended for using TIDE to estimate knockout efficiency in polyclonal populations. However, since ALCAM KO appear to not be efficiently knocked out, it would be important to try multiple independent guides. This may be guide dependent and be an off-target issue with that particular guide, or it could be bona fide biology as the authors suggest.

Reviewer #3

(Remarks to the Author)
In contrast to most AdV Species, Species B use either DSG2 or CD46 as primary receptors. A large number of co-receptors are known to mediate, albeit with less efficiency, or enhance AdV infection. While many of these co-receptors are thought to be virulence factors, there is little evidence for a direct correlation between gene expression and people with infections. The authors use a novel technique to specifically investigate changes in cell surface-associated proteins. They expand on a previous report that used a smaller 'SfCIRSPR' library to identify a novel factors for rhinovirus. With a library >3x larger, several host factors were identified and ALCAM appears to be a putative receptor for Species B AdV that use either DSG2 or CD46. Moreover they identify that endophilin-A3 (Endo-A3), a protein previously implicated in the endocytosis of ALCAM, also plays a role in AdVB infection. The most provocative finding is that ALCAM expression is significantly higher in the people with SCAP associated with AdVB infection over other people with SCAP associated with other infectious agents. There are several outstanding questions.
1. The authors state n = 3 for many experiments. Is this a single experiment run in triplicate or 3 experiments with some number of replicates? Given the small numerical differences for many of the assays (often within 5 – 10% of each other), replication over multiple experiments is essential to distinguish between day-to-day variability and true biological implications. Please add the observed assay variability over multiple runs over multiple days for the RT-qPCR assays in the methods section.
2. The authors state that ALCOM is 'essential' for growth and cell division. Please more clearly define relative survival rate in extended data Fig 7. It seems reduced but not flat. What is your definition of 'essential' based on?
3. The lower 'relative survival rate' indeed makes it tricky for experiments to prove its function as a viral receptor. While the data is relatively convincing, the differences are small. Is this a function of a very low MOI? What percent of cells are GFP positive in the competition experiments? Are the Percentages relative to RFP expression in the control cells? It is unclear how these numbers are calculated. What happens at a higher MOI? While viruses normally have evolved to use proteins that are essential and therefore highly conserved, CHO cells lines have served well as an appropriate cell line for straightforward reconstitution experiment. Have the authors tested CHO cells for ALCAM expression?
4. Are the ALCAM ko cells (and other ko cell lines) a clonal population? Please clarify in the manuscript.
5. What happens to cell growth and division with over expression of ALCAM? This could cause the bounce observed in Fig 2g.
6. If figures are from a previous publication, make sure to reference (e.g. Fig 1a, extended Fig 1a).
7. Do the penton bases of AdV-B and AdV-C interact with the same or different integrins? It is unclear why to start with AdV-C instead of just including data with AdV-B. Does it make a difference for infection?
8. Is Fig 2h a recalculation of data from Fig 2f? If so, please make this clear in the manuscript.
9. Washing generally does not remove bound virus. Normally an enzymatic step is required. What evidence do you have that washing is sufficient? If you do not wash to determine bound virus, what evidence do you have that it is simply virus left in the media and not bound virus? Given the small numerical differences, this could be artifact based on media, washing, and handling of cells.
10. Lines 253 – 257 should be clarified that it is mRNA expression and not protein expression. How do you know that the protein levels, and cell surface levels in particular, are not affected by expression of DSG2 or ALCAM or EndoA3? If protein levels were never investigated, speculation in the discussion is sufficient.
11. Extended data 10 Figure legend Line 876 states 'Analysis of protein' but there is no protein data in the figure. Please correct the title.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Xie & Mei et al. revised their manuscript with additional experiments (e.g. SPR analyses) that overall strengthen conclusions, however certain critical points remain to be revised or insufficiently addressed to publish the manuscript without further revision. In addition, writing quality unfortunately decreased compared to pre-revision manuscript.

Major comments:

#1: In the abstract, the authors did not modify the claim for previous manuscript that the knob-ALCAM interaction has a Kd of 700nM. However, the two additional measures now included the manuscript are slightly different and should be taken into account in the abstract, for example by reporting a dissociation constant of "837nM in average" instead of "approximately 700nM".

#2: The paragraph describing background knowledge on the ALCAM protein (lines 184-190) should feature in the introduction or eventually the discussion section, and not in the results section.

#3: Results section (page 12, second paragraph): The statement that HAdV-B infection is "dependent" on ALCAM should be toned down. It should be rather stated that ALCAM "enhances" or "mediates" (among other factors) the infection. Most importantly, the DSG2-ALCAM double KO now adds the critical information that ALCAM's involvement in infection is dependent on presence of DSG2. This disqualifies ALCAM as a receptor and suggests it is a co-receptor of DSG-2, although authors rightly point out that more information would be necessary to draw this conclusion. Therefore, it would be correct to potentially replace "receptor" by "internalization co-factor" or a similar term in the title, and discuss whether ALCAM is a co-receptor of DSG-2 and what the implications thereof could be, extending their discussion of the DSG2-ALCAM-EndoA3 pathway.

#4: Results section (Page 10): The hypothesis that hamster's ALCAM does not support infection as its human orthologue can be tested with relative ease by transfecting one of the established ALCAM-KO models with hamster's ALCAM and repeating an infection experiment.

Minor comments:

#1: Line 202: Why is the co-culture of WT and KO cells deemed "physiologically relevant"? Relevant for what? Cell types in a patient's body are indeed going to differ in their ALCAM expression levels, but all cells from the same type are expected to have similar expression levels, thus not matching the experimental setting. Please clarify.

#2: Chimeric vectors should not be referred to with the same names as wild-type clinical isolates. For example, name the chimeric vectors "HAdV-C5/B3" etc. to avoid confusions.

#3: The sentence lines 230-232 are confusing. Please reformulate and extend if required. Here it would have helped to accurately name the chimeric vectors. Also it would have helped readers to understand that the described "HAdV-C5 backbone structure" refers to the capsid proteins other than fiber.

#4: The sentence lines 239-240 is unnecessary and grammatically incorrect ("to the unique" should be replaced by "to a unique"). If the authors have no idea what makes HAdV-B35 different, they do not have to speculate.

#5: Line 516 to 520 (materials and methods section): qPCR machines have a background variability much higher than the claimed 2% variation between technical replicates, when considering calculated copy numbers, due to the exponential nature of DNA amplification. Did the authors mean 2% of Cq value? Or did they run numerous qPCR replicates of each sample to decrease variability? Please specify. Same for the 10% rate between biological replicates.

#6: Line 896 (Figure legend 3): what do the authors mean with "the relative cHAdV-B7 infection rate in each group" related to an experiment that does not even feature HAdV-B7? Please reformulate.

#7: Line 903 (Figure legend 3): Please replace "live HAdV-B14" by "wild-type HAdV-B14" or "HAdV-B14 clinical isolate"

Typos:
Line 184, results section: "a type-I transmembrane protein", not "an I-type transmembrane protein"

Line 202, results section: replace " These experiment were conducted under physiologically relavant setting with the mixature of wild type and knockout cells" by " These experiments were conducted under physiologically relevant settings with a mixture of wild type and knockout cells", assuming you keep this sentence (see also minor point #1)

Line 285, results section: "suggesting a", not "suggesting of a"

Line 298, results section: "is dependent on the", not "is dependent on"

Line 302, results section: "nor did DSG-2 knockout affect", not "nor did DSG-2 knockout affected"

Line 376, discussion section: "are worthy", not "is worthy". Besides, as noted in minor point #4, authors may refrain from calling out "unique features of HAdV-B35" if they have no information on what these features are.

Reviewer #2

(Remarks to the Author)
The authors have addressed most of my concerns. I still believe that the magnitude is modest, although reproducible across both chimeric and authentic viral systems. One note is that the data in 3A should be replotted to where the dynamic range is not so small and consistent across each graph. The current way the data is presented appears to exaggerate the differences between samples.

Reviewer #3

(Remarks to the Author)
Please add replicate n, particularly those used in statistical analysis, to figure legends.
Other concerns are addressed.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Xie et al. describe the identification of ALCAM as entry receptor of several species B adenoviruses including pneumonia-associated types. The authors initially found that besides influenza, adenovirus was the second most frequently identified virus infection after analyzing pathogens during severe community-acquired pneumonia (SCAP). They subsequently identified HAdV-B7 as the pathogen which was most frequently detected besides HAdV-B3 and -B55. Using a CRISPR-based library screen to perform a broad knockout of surface receptors, the authors identified ALCAM as entry receptor besides DSG-2. Next Xie and colleagues explored a chimeric HAdV-C5 vector carrying a B7 fiber and they produced ALCAM knockout cells to analyze entry of the GFP encoding chimeric vector. The authors found that DSG-2 influences virus attachment and entry and that ALCAM is solely involved in entry. After generating different variants of fiber and ALCAM encoding plasmids, the authors performed co-IP analyses and identified the fiber domain interacting with ALCAM. Furthermore, the authors show evidence that HAdV-B7 entry depends on the EndoA3 axis. In addition, they performed SPR analyses of ALCAM and HAdV-B7 fiber knob domains and competitions assays to further investigate binding characteristics.
The evidence supporting this claim is overall solid, statistics are rigorously calculated and the authors do not make excessive claims. This is a logical study but for publication further experiments and analyses need to be performed to strengthen the main conclusion. In total, publication of this article can be supported if the authors address the following comments.

Major comments:

• The authors conclude that ALCAM represents a general entry receptor for species B adenoviruses. However, this is not entirely true because Ad35 seems not to be involved and the authors mainly checked chimeric viruses and not wild type viruses. To strengthen this hypothesis the authors should perform experiments with wild type viruses, which can be ordered and/or obtained from reference laboratories.
Response: As suggested, we have examined clinical isolates of HAdV-B14 and -B35 and found that the results were consistent with those of chimeric viruses-*ALCAM* knockout could reduce the infection of B14, but not B35 (Fig. 3e). This result made B35 to be different from other examined HAdV-B (B3, B7, B11, B14, B50) with regard to the usage of ALCAM. We have replaced the term "general" with "widely used" to more accurately described the observation.

For the exception of B35, we currently have no specific explanation that is supported by solid experimental evidence, but speculate that this may be related with some unique feature of B35. We have revised the Results and Discussion to address this point. We would be also happy to take any further suggestions from the reviewer.

• The Kd measured for knob binding to ALCAM should be discussed. Baker et al. (2019, Nature Communications) found a Kd of 66μM between HAdV-B3 knob and DSG-2, suggesting the 700nM value for ALCAM bears witness to a high affinity. However, since the data points do not fit the calculated curve well the actual Kd may be somewhat higher. If this is feasible for the authors, it would be valuable to complete the SPR measurements with HAdV-B7 fiber interactions with DSG-2, or between full virus particles and ALCAM, to verify that the use of purified isolated protein domains does not introduce biases. Furthermore, the authors should include a specificity control in the SPR assay, for an example anti-tag antibodies that should induce a second response peak when added after tagged knobs.

Response: We thank the reviewer for raising this critical question. To thoroughly address the reviewer's concern on the reliability of the SPR experiments, we re-designed and repeated the entire experiments. In the revised manuscript, we have provided additional data with SPR experiments (Fig. 3) as listed below:

1. A biological replicate to the original experiment where ALCAM was ligand and knob was analyte (Extended Data Fig. 12c). This gave a K_D value of 0.849 μM (Fig. 5e), similar to the original number of 0.700 μM.

2. Knob (ligand) against ALCAM (analyte) (Extended Data Fig. 12d) (switching immobilized and flowing molecules). This gave a K_D value of 0.961 μM, similar to the numbers in ALCAM (ligand) against knob (analyte) group.

3. Knob (ligand) against DSG-2 (analyte) (Extended Data Fig. 12e). This gave a K_D value of 2.61 μM (Fig. 5e), several fold greater than the K_D value between ALCAM and knob. These values may be different from the reported values (66 μM) in the literatures (technically it may be hard to keep consistent experimental set up across different labs, especially considering the quality of the proteins from different suppliers or different preparations). Nevertheless we believe that the K_D values across different groups within the present study is comparable due to the consistent quality control.

4. Knob (ligand) against knob (analyte) (Extended Data Fig. 12f). This gave a K_D value of 3.47 μM, which reflected the interactions between oligomerized knob proteins (Van Raaij et al., Nature, 1999).

5. ALCAM (ligand), DSG-2 (ligand) or knob (ligand) against a bacteria-derived nuclease protein LbCas12 (irrelevant negative control) (Extended Data Fig12g-i). The data suggested that ALCAM, DSG-2 or knob did not interact with LbCas12.

We did not use an anti-tag antibody because we found that these antibodies contained glycerol that could interfere with SPR experiments.

We hope that these experiments could address the reviewer's concerns. Nevertheless, we have to admit that the technical perfection of these experiments was largely restricted by the quality and quantity of the purified protein samples from commercial suppliers in a limited time frame.

We have revised the Results, figure legend and Discussion accordingly.

In Figure 1h DSG-2 seems to be a much stronger receptor compared to ALCAM. Have the authors compared binding strength of the HAdV-B7 fiber protein to ALCAM and DSG-2? This could be analyzed by SPR analyses.

Response: Indeed, in Fig. 1h *DSG-2* knockout had stronger effects than *ALCAM* knockout on HAdV-B infection. This was also confirmed by knockout experiments with another sgRNAs (updated Fig. 1h).

In the SPR experiments as described above (see data in Fig. 5), the K_D value between ALCAM of B7 knob was determined to be around 0.7 μM, while the K_D value between DSG-2 of B7 knob was found to be above 2 μM.

The results in knockout experiments and SPR experiments seemed to give different conclusion on the effects of DSG-2 and ALCAM on viral infection. We believe that this discrepancy could be affected by many factors such as the usage of chimeric or native viruses (one notable observation was that ALCAM knockout seemed to have more effects on native viruses than on chimeric viruses).

We have revised the Results, figures/legends and Discussion accordingly.

• Since ALCAM is not involved in attachment, it can be hypothesized to be a co-receptor of DSG-2 or binding factors present in the extracellular matrix. A valuable addition to the paper would be to identify this attachment factor. If this is DSG-2, this could be easily investigated by the authors based on available materials and following the design of the EndoA3 experiment, by knocking down ALCAM in the DSG-2 knockout cell line and measuring if the decrease in infectivity is additive or not.

Response: We thank reviewer for this helpful comment. As suggested, we have conducted *ALCAM* knockdown in wide-type and *DSG-2* knockout HEK-293A cells. The results showed that dual depletion of *DSG-2* and *ALCAM* did not have additive effects on HAdV-B7 infection (Fig. 5b). Thus, DSG-2 and ALCAM should act in the same attachment/entry process for HAdV-B7 infection (due to the redundant loss-of-function). Collectively, the results suggested the existence of DSG-2-ALCAM-EndoA3 axis for HAdV-B attachment and entry (we are still unsure about the detailed relationship between ALCAM and DSG-2 and thus did not make the conclusion of co-receptor). We would be also happy to take further suggestions from the reviewer to make this point clearer.

We have revised the Results, figures/legends and Discussion accordingly.

• Why do the authors assume that DSG-2 expression levels should be increased in SCAP patients? Is there any evidence in the literature that DSG-2 expression levels are enhanced after infection with adenovirus utilizing DSG-2 as entry receptor? Please clarify this in the discussion.

Response: We apologize for the confusion. Our initial assumption before performing the CRISPR screen was to expect differential expression level of DSG-2 in SCAP patients that may confer patients different susceptibility to HAdV-B

infection. We have revised the text and discussion to clarify this.

• Extended data Fig. 7f: The ALCAM band in the Western blot analyses seems to be very weak. Did the authors check ALCAM expression levels on different cell lines from different origin? Have the authors checked for cell lines which naturally display low or high expression levels of ALCAM? These cell lines could be also used to study cell entry and would strengthen the data set.

Response: In Extended Data Fig. 7f, the *ALCAM* overexpression was conducted with a strong promoter EF-1α, likely resulting in much higher expression than the endogenous expression. We have provided flow cytometry analysis of cell surface *ALCAM* expression as further evidence (Extended Data Fig. 7g).

We examined several cell lines for *ALCAM* expression (Extended Data Fig. 9a) and found that HeLa cells had notably lower expression. We performed HAdV-B7 infection in these cells and found that HeLa cells had much lower viral titer than other cells (Extended Data Fig. 9b). We thus overexpressed *ALCAM* in HeLa cells (Extended Data Fig. 9c-d) to conduct gain-of-function analysis. It was found that *ALCAM* overexpression could increase the infection of B7 chimeric and wild-type viruses (Fig. 3h-i and Extended Data Fig. 9e). These data further validated the function of ALCAM in HAdV-B infection.

One interesting observation was that although ALCAM low expression appeared to result in low HAdV-B infection, HAdV-B infection did not 100% align with ALCAM expression (A549 showed notably higher viral infection but exhibited similar level of ALCAM expression as compared to other cells) (Extended Data Fig. 9a-b). We have expanded Discussion on this part.

• Discussion section: Maybe I missed that information but in my opinion, the authors should go into more detail about what is known regarding ALCAM (e.g. expression pattern in vivo, function, which cells express ALCAM). Moreover, the authors should discuss potentially other factors involved in uptake as for instance co-receptor usage or other factors which may influence transduction efficiencies as well.

Response: We have added the description of ALCAM in the Results as suggested.

Minor comments:

• Introduction section, line 56: There are >100 adenovirus types. Please correct.

Response: The number has been corrected.

• The fact that HAdV-C5 infectivity is not decreased by ALCAM knockout, and even increased, is an important control and should be discussed. It shows that ALCAM effect on species B adenoviruses is directly related to the virus infection itself and not to indirect influences through changes in cell growth, morphology or viability; that the fiber protein is indeed responsible for these effects, and not the Ad5 backbone used in

this study; and questions whether HAdV-B35 infection is truly independent of ALCAM or if the lack of significance stems from opposite effects of ALCAM deletion on virus entry versus indirect cell effects.

Response: We thank the helpful comments from the reviewer and have expanded discussion on HAdV-C5 as a negative control.

With regard to HAdV-B35 infection, we have provided additional experimental data showing that *ALCAM* knockout did not significantly affect the infection of clinical isolate of HAdV-B35 (Fig. 3e). Due to the time limitation, we did not include experiments in this revision to discriminate the direct effects of ALCAM depletion on virus infection and its indirect effects on cell proliferation.

• In figure 2, the effect of ALCAM knockout seems much more limited than that of its knockdown. Does this suggest ALCAM influences much more viability/growth than infection? The magnitude of ALCAM's involvement in infection in physiologically-relevant settings should be discussed.

Response: We agree that the effects of *ALCAM* knockout may be involved. In addition to this, we believe that other factors such as the use of single knockout clones or mixed population, the use of chimeric viruses or native viruses may be also involved. We have revised the Results and Discussion to address this point.

• Is it correct that the "ALCAM KO" cell line is derived from HEK-293T and "ALCAM-/-" from HEK-293A? Please make it more explicit, the presence of both cell lines may confuse readers.

Response: We apologize for the confusion. We used "KO mix" to reference knockout on mixed population and *ALCAM*$^{-/-}$ for knockout single clones with both alleles of *ALCAM* containing out-of-frame indels. KO and *ALCAM*$^{-/-}$ were not meant to be referencing different cell lines. We have revised the legend of Fig. 2f to clarify this.

• In figure 4, the signification of abbreviations on the left of the pictures (e.g. "WCL") should be explained.

Response: We have revised the legend to indicate its meaning.

• Figure 4f suggests that isolated ALCAM C domain binds to the shaft as well. Please discuss.

Response: We have provided discussion in the Results to address this point.

• Extended figure 9 suggests that DSG-2 binds preferentially to monomers, then to trimers, then to dimers; while ALCAM has apparently a preference for dimers and

hardly binds to trimers. The authors should discuss if this may be explained by technical biases or is representative of physiological conditions. One may hypothesize that the scarcity of intact fiber trimer binding to ALCAM explains its lack of involvement in attachment, while it becomes involved once the fibers start dissociating at the entry step.
Response: We apologize for the confusion. The Co-IP analysis was performed with denatured gel and thus the blotted bands with different molecular weights were unlikely to be oligomerized fiber. Rather, we believe that the smaller bands corresponded to fragmental proteins such as different domains of fiber (shaft, knob, tail or combinations).

We have revised the text and figure legend (figure re-numbered to be Extended Data Fig. 10) to clarify this point.

• Figure 2: Was the surface expression of ALCAM after knockdown checked by flow cytometry and how do the author explain the remaining transduction efficiencies of more than 20% (Fig. 2g)?
Response: As suggested, we have performed flow cytometry analysis of cell surface ALCAM (Extended Data Fig. 7g). It seemed that in ALCAM$^{-/-}$ cells, little expression could be detected. However, as stated above, HAdV-B seemed to have alternative pathway other than DSG-2-ALCAM-EndoA3 for attachment or entry (Fig. 5a-b). We think this could explain the residual transduction efficiency in Fig. 2g.

• Extended data figure 2: Did the authors check DSG-2 and CAR receptor expression on the cell surface by flow cytometry before and after knockout? How do the authors explain that there are still ~20% transduction efficiencies with HAdV-C5?
Response: As suggested, we have performed flow cytometry analysis of cell surface expression of CAR and DSG-2 (Extended Data Fig. 3b-c). There seemed to be residual expression on cell surface in knockout cells. This could be because the knockout was performed on mixed population without isolation of single clones. Thus we believe that the ~20% transduction efficiency of HAdV-C5 on *CAR* knockout cells arose from the residual expression of CAR in the mixed population

We have revised the Results to address this point.

• Cell clones for knockout cells were selected and single clones were chosen. However, how where the single cell clones selected and how and which clones were chosen for further use? By expression levels of the receptor or just by genome analyses?
Response: The single clones were mainly selected based on the results of gene modifications. Only clones with both alleles containing out-of-frame indels were selected. In addition to genomic analyses (Sanger sequencing of PCR-amplified alleles), WB and flow cytometry analysis could be also used to characterize the isolated clones for expression of target protein.

We have revised the Methods to clarify this point.

Spelling mistakes
• Line 64: Introduction: “the second most prevalent viral pathogen” (“most” is missing)
Response: We have corrected the typo.

• Line 77: “cellular proteins may be involved in the infection”
Response: We have corrected the typo.

• Line 190: “ALCAM knockout cells exhibited reduced rate of infection” (“the” to be removed)
Response: We have corrected this typo.

• Line 223: “ALCAM KO cells were incubated”, not “was”
Response: We have corrected this typo.

• Results section: “receptor usage”, not “receptor utility” (three occurrences)
Response: We have corrected the typo.

• Line 312: The sentence should be corrected. I think there is a “with” missing.
Response: We have corrected the sentence.

• Line 365: Methods: with “Cells of more than 500-fold coverage of the library size“, do the authors mean that the number of collected cells is 500 times higher than the number of sgRNAs in the libraries?
Response: Yes. We have revised the text to clarify this.

• Line 480: “with a pore size of 0.45 μm”, not μM
Response: We have corrected the typo.

We are grateful to the reviewer for these helpful comments.

Reviewer #2 (Remarks to the Author):

In “ALCAM is A Functional Entry Receptor for Severe Community Acquired Pneumonia-associated Human Adenovirus Species B” Xie and Mei et al perform a targeted CRISPR KO screen to identify surface proteins that are necessary for Human Adenovirus B (HAdV-B) entry. Authors demonstrate that ALCAM deficiency provides a modest deficiency in HAdV-B infection likely at the step of viral internalization. A direct interaction between ALCAM and the HAdV knob was mapped. While the topic

is of interest, the data is far from convincing that ALCAM is an entry receptor. Rather the data points to a quite modest facilitation of viral internalization by ALCAM1. Overall, the mechanistic data lacks rigor. A point by point critique follows:

Major Point

1. The phenotypic magnitude isn't compelling for a receptor. For example, in many panels the difference between WT or non-targeted samples is only 10% (e.g. Figures 2H, 3A, and throughout). This isn't consistent with other reports of HAdV receptors (e.g. PMID: 21151137). Furthermore, the presentation of the data with graphs starting at 85 or 90 percent of a normalized value casts doubt as to the role of this molecule in HAdV infection.

Response: We agree with the reviewer that in some experiments, particularly those of chimeric viruses, the phenotypes between WT and *ALCAM* knockout cells are small (though with statistical significance). We believe that this could be explained by the non-native nature of the chimeric viruses. The phenotypes with native viruses (clinical isolates) are much more notable (Fig. 3c-f, Fig. 4c, Fig. 5a). To further address the reviewer's concern, we performed more phenotypical experiments with native viruses (clinical isolates) (see new data in Fig. 3e, Fig. 5a-b). In addition, we have overexpressed *ALCAM* in HeLa cells, which had low endogenous *ALCAM* expression (Extended Data Fig. 9), and found that *ALCAM*-overexpressed HeLa cells were more susceptible to HAdV-B infection (Fig. 3h-i).

We hope that these additional data could address the reviewer's concerns on the phenotypical experiments. We would be also happy to take any further suggestions from the reviewer.

2. The authors conflate "entry" and "internalization". In figure 4, the authors are actually measuring viral internalization. An entry receptor would be considered to help the virus uncoat, fuse, or escape the endosome. The data presented suggests a modest decrease in internalization rather than these steps.

Response: We thank the reviewer for raising this point. This is indeed a critical question for this study. Since we have included many new data in the revised manuscript, we would like to recapitulate the relevant data in the original and revised manuscripts to discuss with the reviewer how to best define the role of ALCAM:

First of all, we agree with the reviewer that the experiment in Fig. 4a-c was largely related to the internalization process. The experiment was set as below: cells were treated with DPBS on ice for 1 h and with DPBS at 37 ℃ for 45 min. The cells were then treated with trypsin to remove surface-bound virus and then harvested and lyszed for analysis of total virus inside cells. As this process did not distinguish the endosomal and cytoplasmic compartments, the viruses we were looking at were in fact mixed population in endosome and cytoplasm. Because the time was limited to 45 min (probably not sufficient to allow the virus to escape

from the endosome), one would expect that the majority of these viruses were still in the endosomes, just as the reviewer pointed out.

Second, as shown in Fig. 4a-c, unlike DSG-2, ALCAM did not contribute to virus attachment (which was in fact a bit surprising to us given its widespread functions in cell adhesion, growth and migration) but act at subsequent processes. This suggested that ALCAM participated in the process of endocytosis or entry/endosomal escape, or even both.

Third, according to the SPR data (new data provided in Fig. 5), ALCAM had direct interaction with HAdV-B7 knob, which seemed to be stronger than B7 knob and DSG-2 interaction (Fig. 5e).

For the above reasons, we propose to make the following revisions: 1) rather than emphasizing "entry", we define ALCAM as a widely used "receptor", which is supported by both the cell culture and SPR experiments; 2) clarify that Fig. 4 is more related to virus internalization. We have made revisions in the title, text, figure legends and Methods accordingly.

We hope that these revisions can address the reviewer's concerns on this issue. We would be also happy to take any further suggestions from the reviewer.

3. Throughout the manuscript the authors use a t-test when comparing more than two groups. A statistical test that accounts for multiple comparisons (e.g. ANOVA) is absolutely necessary especially given the modest magnitude.

Response: We have carefully reviewed the statistically analyses throughout the manuscript and indeed identified cases where ANOVA, rather than t test, should be used. We have changed these analyses to ANOVA and revised the figures, figure legends and Methods accordingly.

We thank the reviewer for this helpful comment.

4. Connections between ALCAM expression and SCAP are tenuous and don't provide insight into the biology. It is unclear why SCAP patients would have different expression of a HAdV receptor. Overall these connections detract from the main story.

Response: Our initial attempt was to explore possible association between the expression of receptors and AdV infection in SCAP patients. As suggested, we have revised the text to clarify this and to ensure the acquired data are focused on the main theme of the study.

5. Figure 4D-4F, many of the deletion mutants still interact. Thus it is imperative to include a negative control protein for the interactions studies (e.g. MBP or GFP or even an HAdV-C5 fiber). It is quite likely that these are sticky proteins that have non-specific interactions via Co-IP

Response: We thank the reviewer for raising this critical question. We tend to have the same speculation that ALCAM and HAdV fiber are sticky proteins. Specifically for ALCAM, its V- and C-domains are composed of several

structurally similar immunoglobulin subunits. It has been reported that ALCAM can mediate both heterophilic (ALCAM-CD6) and homophilic (ALCAM-ALCAM) cell-cell interactions and is responsible for cellular growth and migration (Kempen et al., 2001, J Biol Chem; Cárdenas Delgado et al., 2011, FASEB J). We have revised the results to provide more information on ALCAM.

With regard to the negative control, we have included an EGFP control in Co-IP experiment (Extended Data Fig. 10b) to exclude possible non-specific interactions in the Co-IP analyses.

Minor Points

1. The writing could be improved in many areas.

Response: We have extensively revised the text and corrected several typos.

2. The authors are commended for using TIDE to estimate knockout efficiency in polyclonal populations. However, since ALCAM KO appear to not be efficiently knocked out, it would be important to try multiple independent guides. This may be guide dependent and be an off-target issue with that particular guide, or it could be bona fide biology as the authors suggest.

Response: As suggested, we have provided the results of another sgRNA (Fig. 1h). Different sgRNAs resulted in consistent phenotypes.

Reviewer #3 (Remarks to the Author):

In contrast to most AdV Species, Species B use either DSG2 or CD46 as primary receptors. A large number of co-receptors are known to mediate, albeit with less efficiency, or enhance AdV infection. While many of these co-receptors are thought to be virulence factors, there is little evidence for a direct correlation between gene expression and people with infections. The authors use a novel technique to specifically investigate changes in cell surface-associated proteins. They expand on a previous report that used a smaller 'SfCIRSPR' library to identify a novel factors for rhinovirus. With a library >3x larger, several host factors were identified and ALCAM appears to be a putative receptor for Species B AdV that use either DSG2 or CD46. Moreover they identify that endophilin-A3 (Endo-A3), a protein previously implicated in the endocytosis of ALCAM, also plays a role in AdVB infection. The most provocative finding is that ALCAM expression is significantly higher in the people with SCAP associated with AdVB infection over other people with SCAP associated with other infectious agents.

There are several outstanding questions.

1. The authors state n = 3 for many experiments. Is this a single experiment run in triplicate or 3 experiments with some number of replicates? Given the small numerical differences for many of the assays (often within 5 – 10% of each other), replication over

multiple experiments is essential to distinguish between day-to-day variability and true biological implications. Please add the observed assay variability over multiple runs over multiple days for the RT-qPCR assays in the methods section.

Response: We thank the reviewer for this helpful comment. These replicates are all biological replicates that reflected the day-to-day or person-to-person variations. In the RT-qPCR, we typically included two technical replicates for each biological replicate. The variations between technical replicates were mostly below 2% and the mean values of the technical replicates were recorded. The variations between biological replicates could be typically controlled to be within 10%. We have revised the Method as suggested to clearly indicate this.

2. The authors state that ALCOM is 'essential' for growth and cell division. Please more clearly define relative survival rate in extended data Fig 7. It seems reduced but not flat. What is your definition of 'essential' based on?

Response: We thank the reviewer for raising this question. This is indeed a confusing description. We claimed ALCAM as "essential gene" following the concepts in gene essentiality studies (Gönen et al., 2017, Cell Syst) where the genes are defined as "essential" when their depletion inhibit or abolish cell growth/survival and as "non-essential" otherwise. This concept is indeed a bit different from the strict cell biology concept of "essential" where knockout of such essential genes will eliminate cell growth.

To avoid further confusion, we have revised the text to clarify the effects of ALCAM on cell proliferation.

3. The lower 'relative survival rate' indeed makes it tricky for experiments to prove its function as a viral receptor. While the data is relatively convincing, the differences are small. Is this a function of a very low MOI? What percent of cells are GFP positive in the competition experiments? Are the Percentages relative to RFP expression in the control cells? It is unclear how these numbers are calculated. What happens at a higher MOI? While viruses normally have evolved to use proteins that are essential and therefore highly conserved, CHO cells lines have served well as an appropriate cell line for straightforward reconstitution experiment. Have the authors tested CHO cells for ALCAM expression?

Response: We agree with the reviewer that some phenotypical experiments indeed gave small difference between wild type and *ALCAM* knockout, particularly in the cases of chimeric viruses. We believe that this could be explained by the non-native nature of the chimeric viruses. The phenotypes with wild-type viruses (clinical isolates) are much more notable (Fig. 3c-f, Fig. 4c, Fig. 5a).

To further address the reviewer's concern, we performed more phenotypical experiments with native viruses (clinical isolates) (see new data in Fig. 3e, Fig. 5b). In addition, we have overexpressed *ALCAM* in HeLa cells, which had low endogenous *ALCAM* expression (Extended Data Fig. 9), and found that

overexpressed HeLa cells were more susceptible to HAdV-B infection (Fig. 3g-i). Therefore, the function of ALCAM is well supported.

In Fig. 3 where small difference was observed as the reviewer pointed out, the MOI of HAdV was low, approximately 0.002 (see Method section). While in some other studies where relatively larger difference was observed (Fig. 4a-c), much higher virus titers (MOI of 20) were used.

With regard to the competition experiments, GFP-positive population was around 80% (Fig. 2e-f). In test groups (*ALCAM* knockout), GFP positive rate was calculated based on the RFP- population. In the control groups (wild type), GFP positive rate was calculated based on the RFP+ population. We have revised the Methods to provide more experimental details.

For the CHO cell experiment, we have analyzed its *ALCAM* expression and susceptibility to HAdV-B infection. It was found that CHO-K1 cells with high *ALCAM* expression were barely infected by HAdV-B (Extended Data Fig. 9f-h). This suggested that CHO ALCAM might not support HAdV-B infection as human ALCAM did.

4. Are the ALCAM ko cells (and other ko cell lines) a clonal population? Please clarify in the manuscript.

Response: *ALCAM* KO mix was mixed population without isolation of single clones. *ALCAM*$^{-/-}$ cells were isolated single clones. We have revised the figure legend and Methods to clarify this.

5. What happens to cell growth and division with over expression of ALCAM? This could cause the bounce observed in Fig 2g.

Response: We have quantified the proliferation of wild-type, *ALCAM*-/- and overexpression rescued *ALCAM*-/- cells. *ALCAM* knockout slightly decreased cell proliferation and *ALCAM* overexpression in *ALCAM*-/- cells slightly increased cell proliferation (to a level more close to proliferation in wild type) (Extended Data Fig. 7h). The effects of *ALCAM* knockout or overexpression on HAdV-B infection was more notable. Thus, we believe that the observed effects of ALCAM on virus infection was direct effects, rather than indirect effects from cell proliferation.

6. If figures are from a previous publication, make sure to reference (e.g. Fig 1a, extended Fig 1a).

Response: The data were extracted from a previous publication and re-analyzed and figures re-made. We have included the reference as suggested.

7. Do the penton bases of AdV-B and AdV-C interact with the same or different integrins? It is unclear why to start with AdV-C instead of just including data with AdV-B. Does it make a difference for infection?

Response: HAdV-C uses CAR as the receptor and HAdV-B use DSG-2 or CD46 as the receptor. Since our chimeric virus was constructed on the basis of AdV-C5 (C5 penton base + B7 fiber), we wanted to make sure that the chimeric viruses use HAdV-B receptor rather than C5 receptor (in other words, the B fiber is functional). Therefore, HAdV-C was used as an important control to assess whether the chimeric viruses were generated successfully to mimic the behavior of HAdV-B.

We have revised the text in the Results to clarify this.

8. Is Fig 2h a recalculation of data from Fig 2f? If so, please make this clear in the manuscript.

Response: We apologize for the confusion. The data of Fig. 2e-f were from *ALCAM* knockout mixed population (without isolation of single clones), and the data of Fig. 2g-h were from *ALCAM* knockout single clones.

We have revised figure legend to clarify this.

9. Washing generally does not remove bound virus. Normally an enzymatic step is required. What evidence do you have that washing is sufficient? If you do not wash to determine bound virus, what evidence do you have that it is simply virus left in the media and not bound virus? Given the small numerical differences, this could be artifact based on media, washing, and handling of cells.

Response: The attachment and internalization assays were set in the rationale below:

-attachment. Virus was incubated with cells on ice for 1 h (at this stage, virus will only bind to but not internalize into cells). Then the virus-containing medium was removed and cells were washed extensively with PBS (this step removed the residual viruses in the medium and the viruses non-specifically bound to cells, but left behind the viruses specifically bound to cellular receptors). In this assay, we did not intend to remove surface-bound viruses as this was what we looked for.

-internalization. After incubation on ice for 1 h, virus internalization was initiated by incubating at 37 degree for 45 min. The cells were washed extensively as described above, with the addition of a trypsin treatment. In this assay, we aimed to remove all surface-bound viruses as we were looking for internalized viruses only.

We have revised the Methods to clarify the experimental details.

10. Lines 253 – 257 should be clarified that it is mRNA expression and not protein expression. How do you know that the protein levels, and cell surface levels in particular, are not affected by expression of DSG2 or ALCAM or EndoA3? If protein

levels were never investigated, speculation in the discussion is sufficient.
Response: We thank the reviewer for this helpful comment. We have quantified the cell surface expression of DSG-2 expression on *ALCAM* knockout cells and ALCAM expression on *DSG-2* knockout cells by flow cytometry and found that they were not affected by each other (Extended Data Fig. 11c-d).

11. Extended data 10 Figure legend Line 876 states 'Analysis of protein' but there is no protein data in the figure. Please correct the title.
Response: We have corrected the typo.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #1 (Remarks to the Author):

Xie & Mei et al. revised their manuscript with additional experiments (e.g. SPR analyses) that overall strengthen conclusions, however certain critical points remain to be revised or insufficiently addressed to publish the manuscript without further revision. In addition, writing quality unfortunately decreased compared to pre-revision manuscript.

Major comments:

#1: In the abstract, the authors did not modify the claim for previous manuscript that the knob-ALCAM interaction has a Kd of 700nM. However, the two additional measures now included the manuscript are slightly different and should be taken into account in the abstract, for example by reporting a dissociation constant of "837nM in average" instead of "approximately 700nM".
Response: We thank the reviewer for pointing this out and have made the modification as suggested.

#2: The paragraph describing background knowledge on the ALCAM protein (lines 184-190) should feature in the introduction or eventually the discussion section, and not in the results section.
Response: We have made the changes as suggested.

#3: Results section (page 12, second paragraph): The statement that HAdV-B infection is "dependent" on ALCAM should be toned down. It should be rather stated that ALCAM "enhances" or "mediates" (among other factors) the infection. Most importantly, the DSG2-ALCAM double KO now adds the critical information that ALCAM's involvement in infection is dependent on presence of DSG2. This disqualifies ALCAM as a receptor and suggests it is a co-receptor of DSG-2, although authors rightly point out that more information would be necessary to draw this conclusion. Therefore, it would be correct to potentially replace "receptor" by "internalization co-factor" or a similar term in the title, and discuss whether ALCAM is a co-receptor of DSG-2 and what the implications thereof could be, extending their discussion of the DSG2-ALCAM-EndoA3 pathway.
Response: We have made the revision as suggested.

#4: Results section (Page 10): The hypothesis that hamster's ALCAM does not support infection as its human orthologue can be tested with relative ease by transfecting one of the established ALCAM-KO models with hamster's ALCAM and repeating an infection experiment.
Response: We have overexpressed hamster *Alcam* in ALCAM$^{-/-}$ HEK-293A cells. The results showed that hamster ALCAM did not support HAdV-B infection as human ALCAM did (Extended Data Fig. 9i-j).

Minor comments:

#1: Line 202: Why is the co-culture of WT and KO cells deemed "physiologically relevant"? Relevant for what? Cell types in a patient's body are indeed going to differ in their ALCAM expression levels, but all cells from the same type are expected to have similar expression levels, thus not matching the experimental setting. Please clarify.
Response: We apologize for the confusion. WT and KO cells were co-cultured in the same well just so that they will have identical micro-environment (for example, Knockout of a surface protein may alter the content of cellular matrix). We have revised the text to clarify this point.

#2: Chimeric vectors should not be referred to with the same names as wild-type clinical isolates. For example, name the chimeric vectors "HAdV-C5/B3" etc. to avoid confusions.
Response: We have revised the text as suggested.

#3: The sentence lines 230-232 are confusing. Please reformulate and extend if required. Here it would have helped to accurately name the chimeric vectors. Also it would have helped readers to understand that the described "HAdV-C5 backbone structure" refers to the capsid proteins other than fiber.
Response: We have revised the backbone structure specifically to "capsid" as suggested.

#4: The sentence lines 239-240 is unnecessary and grammatically incorrect ("to the unique" should be replaced by "to a unique"). If the authors have no idea what makes HAdV-B35 different, they do not have to speculate.
Response: As suggested, we have deleted the sentence to avoid confusion.

#5: Line 516 to 520 (materials and methods section): qPCR machines have a background variability much higher than the claimed 2% variation between technical replicates, when considering calculated copy numbers, due to the exponential nature of DNA amplification. Did the authors mean 2% of Cq value? Or did they run numerous qPCR replicates of each sample to decrease variability? Please specify. Same for the 10% rate between biological replicates.
Response: We apologize for the confusion. Indeed, we run multiple qPCR replicates. The 2% variation refers to Ct (cycle of threshold) values. We have revised the text of this part as suggested.

#6: Line 896 (Figure legend 3): what do the authors mean with "the relative cHAdV-B7 infection rate in each group" related to an experiment that does not even feature HAdV-B7? Please reformulate.
Response: We have revised the legend to avoid confusion.

#7: Line 903 (Figure legend 3): Please replace "live HAdV-B14" by "wild-type HAdV-B14" or "HAdV-B14 clinical isolate"
Response: We have revised the legend as suggested.

Typos:
Line 184, results section: "a type-I transmembrane protein", not "an I-type transmembrane protein"
Response：We have corrected the typo.

Line 202, results section: replace " These experiment were conducted under physiologically relavant setting with the mixature of wild type and knockout cells" by " These experiments were conducted under physiologically relevant settings with a mixture of wild type and knockout cells", assuming you keep this sentence (see also minor point #1)
Response：We have corrected the typo.

Line 285, results section: "suggesting a", not "suggesting of a"
Response: We have corrected the typo.

Line 298, results section: "is dependent on the", not "is dependent on"
Response: We have corrected the typo.

Line 302, results section: "nor did DSG-2 knockout affect", not "nor did DSG-2 knockout affected"
Response：We have corrected the typo.

Line 376, discussion section: "are worthy", not "is worthy". Besides, as noted in minor point #4, authors may refrain from calling out "unique features of HAdV-B35" if they have no information on what these features are.
Response: We have revised the text as suggested (speculation on HAdV-B35 is deleted).

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my concerns. I still believe that the magnitude is modest, although reproducible across both chimeric and authentic viral systems. One note is that the data in 3A should be replotted to where the dynamic range is not so small and consistent across each graph. The current way the data is presented appears to exaggerate the differences between samples.
Response: We have replotted Figure 3A as suggested.

Reviewer #3 (Remarks to the Author):

Please add replicate n, particularly those used in statistical analysis, to figure legends. Other concerns are addressed.

Response: We have revised the text as suggested.