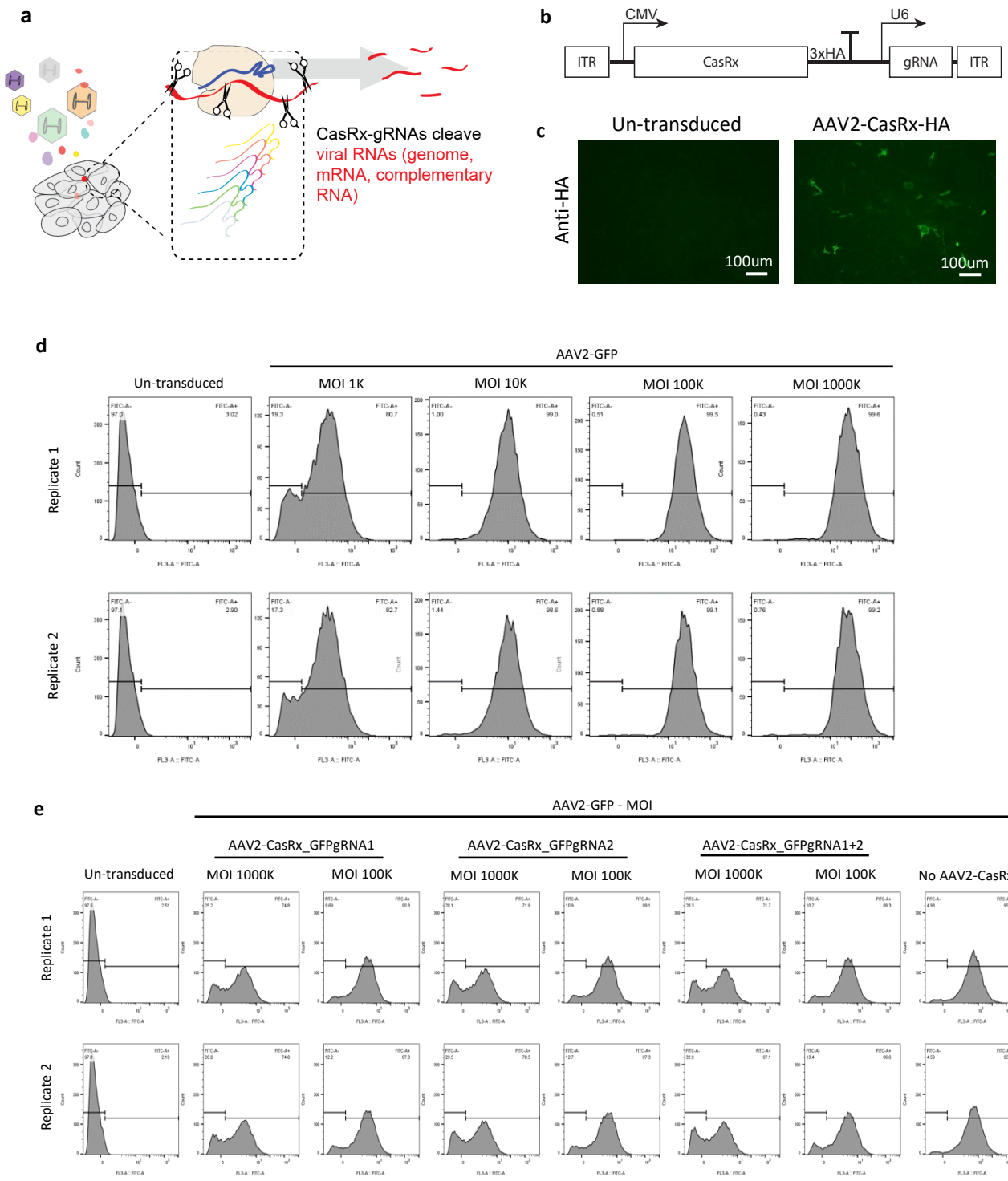
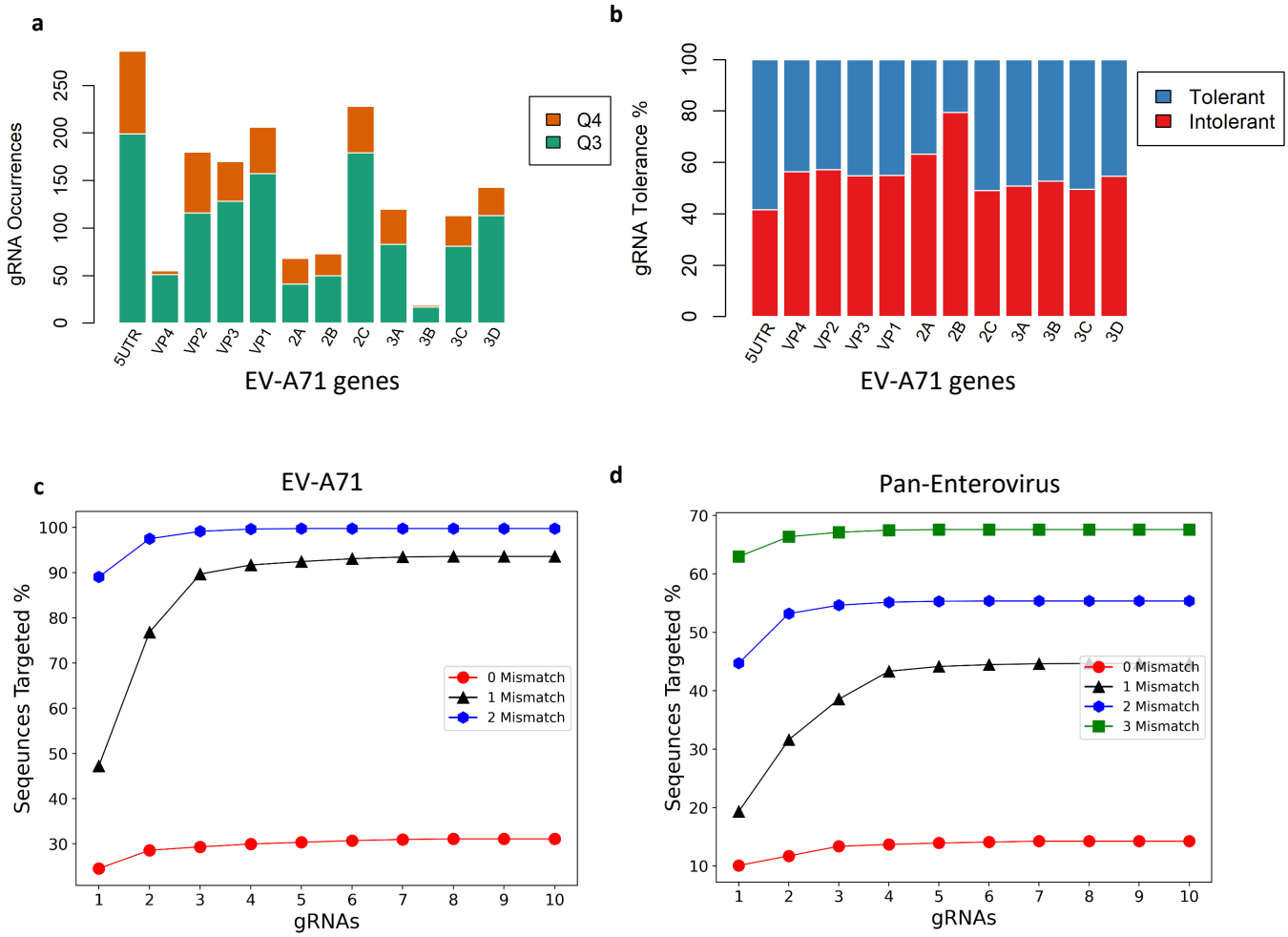
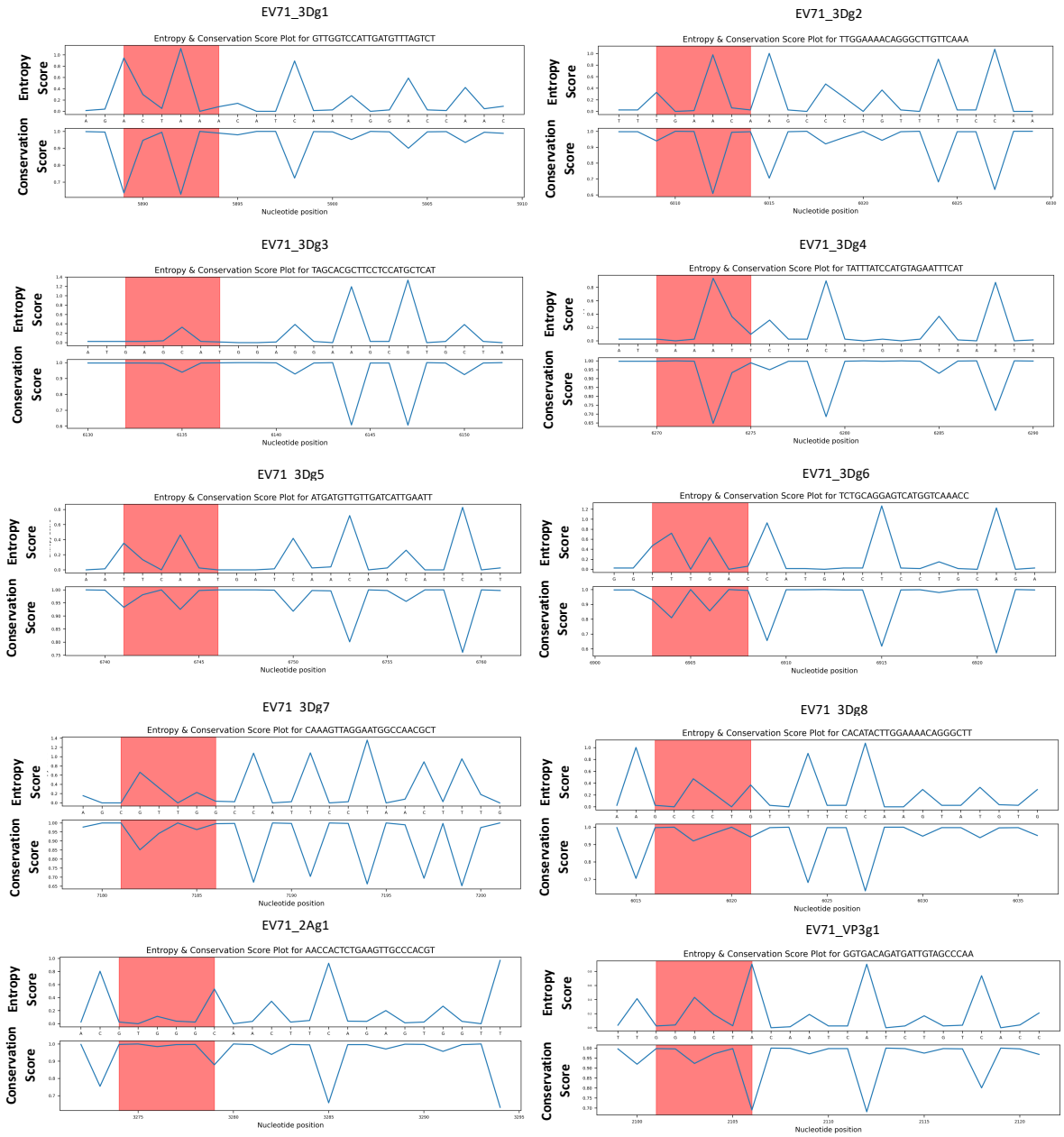


Supplementary Figure 1. **Design of new antiviral AAV-CRISPR-CasRx therapeutics for RNA virus targeting. a.** Schematics of CRISPR-CasRx antiviral activity on RNA viruses. Positive strand RNA viruses such as EV-A71 release their genomes upon entry into the host cell, which serve as templates for negative-strand genomic and subgenomic RNA, which in turn produce more copies of the positive-strand viral genome and viral mRNA. AAV-delivered CasRx and guide RNAs (gRNAs) expressed in the EV-A71-infected cell can specifically target and cleave both the viral genome and viral mRNA to disrupt viral replication and viral functions. **b.** Schematic of the AAV therapeutic cargo. Between the AAV Inverted Terminal

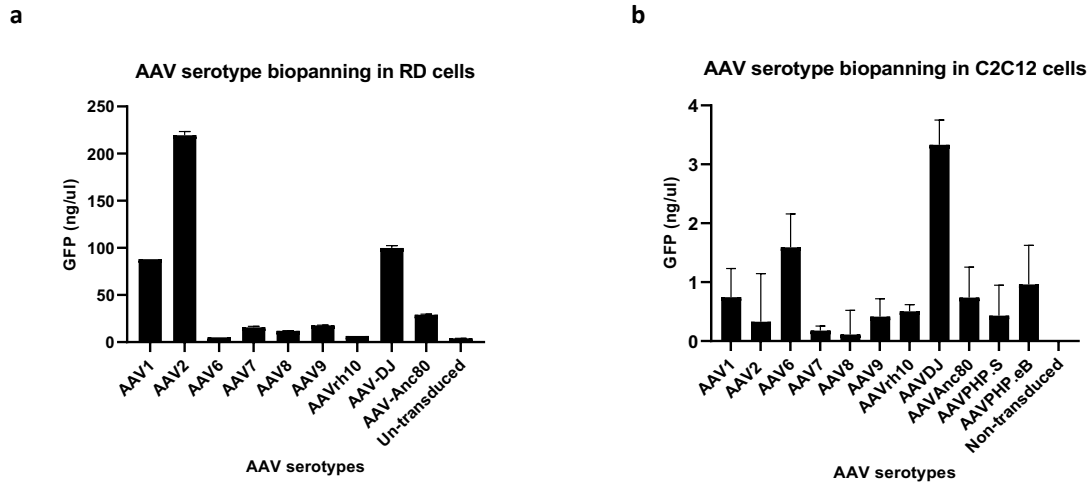
Repeats (ITRs), the CMV promoter drives the expression of CasRx with a 3xHA tag and without Nuclear Localization Signal (NLS), followed by a rabbit polyadenylation sequence. The U6 promoter drives transcription of the gRNA. **c.** CasRx-3xHA is expressed in RD cells. Cells were stained for CasRx-3xHA by immunocytochemistry. Magnification 20X; Scale bar, 100μm. **d**. Titration of AAV2-GFP transduction for GFP expression in immortalized human muscle RD cells. 10K RD cells were plated in a 48-well plate and transduced with AAV2-GFP at MOI of 1K, 10K, 100K and 1000K with untransduced negative controls. **e.** Validation of AAVs with new cargo plasmid bearing CasRx and gRNA**.** Human muscle immortalized RD cells were transduced with AAV2-GFP at MOI of 10K. The GFP knockdown efficiency of gRNA1 only, gRNA2 only, and gRNA1+gRNA2 are tested by treating the GFP-expressing RD cells with AAV2-CasRx bearing the GFP-targeting gRNAs at MOI of 100K or MOI of 1000K for 72h. Control showed no GFP expression (leftmost column) while AAV2-GFP only control showed more than 96% of cells expressing GFP (rightmost column). Number of GFP+ cells decreased when cells were treated with GFPgRNA1 at MOI of 100K or 1000K, GFPgRNA2 at MOI of 100K or 1000K, or GFPgRNA1+2 at MOI of 100K or 1000K.



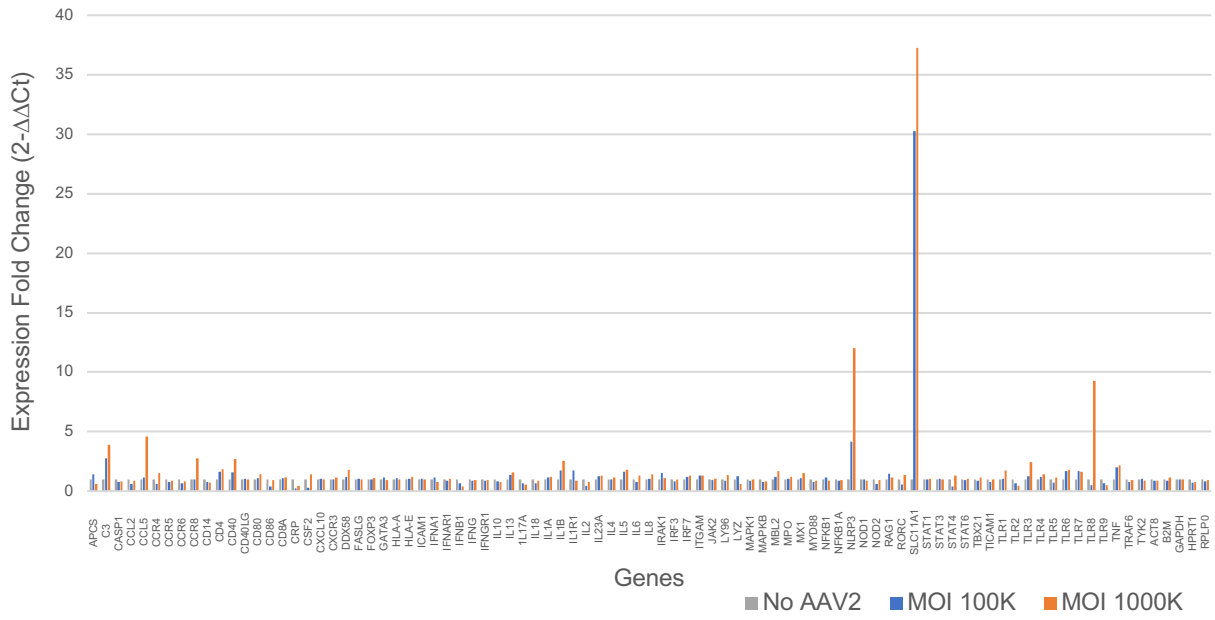
Supplementary Figure 2. Candidate gRNAs targeting Enterovirus-A71. a. Total number of 3rd and 4th Quartile gRNAs with at most 3 mismatches against each gene. **b.** The percentage of gRNAs with mismatches within the intolerant region (15-21 bp of the gRNAs) per gene. **c.** Predicted minimum number of gRNAs required to target all analyzed EV-A71 genomes with allowances for mismatches (n = 736). gRNAs with mutations in the intolerant region are ignored in the analysis. **d.** Predicted minimum number of gRNAs required to target all analyzed EV-A71 (n = 736), Enterovirus-B (n = 414) and Enterovirus-C (n = 735) genomes with allowances for mismatches.



Supplementary Figure 3. Entropies and conservation scoring for each of the selected gRNAs. Each of the selected gRNA was analyzed for entropy score and conservation score, which are used to calculate the final gRNA score (Table 1). The intolerant region of gRNA (*peach*) is most sensitive to target:gRNA mismatches.



Supplementary Figure 4. Bio-panning to identify efficient AAV serotypes. a. The human skeletal muscle is a target organ that supports EV-A71 infection and replication. Using the human RD rhabdomyosarcoma cell line (ATCC), we performed an AAV panel bio-panning experiment to determine the AAV serotype(s) that most efficiently transduce human muscle cells. 10K cells were plated into each well of a 96-well plate and transduced with each serotype of AAV at MOI of 100K and the expression of GFP protein was quantitated at 72h post-transduction. Experiments were performed in duplicates. **b.** Bio-panning was similarly conducted in the mouse C2C12 skeletal muscle cells. Experiments were performed in duplicates.



Supplementary Figure 5. Human innate and adaptive immune response profiling in AAV2-transduced human RD cells. In each 6-well dish, 1 million human skeletal RD cells were seeded and transduced with AAV2 serotype at MOI of 100K and 1000K. RNA of the transduced cells was harvested at 72 hrs post-transduction using the RNeasy universal plus mini kit (Qiagen). cDNA was synthesized and the immune response gene panel was quantitated using the RT2 profiling kit (Qiagen). The result showed that there is minimal antiviral cellular response in the AAV2-transduced RD cells with only two genes (NLRP3 and SLC11A1) significantly upregulated in a dose-dependent manner. NLRP3 and SLC11A1 are both involved in macrophage activation or recruitment but have not been shown to exhibit intracellular antiviral activity.

H strain (AY053402.1)

```
ID:3Dg1 Variant:AY053402.1 Pos:5974
Original gRNA [EV-A71]:
GTTGGTCCATTGATGTTTAGTCT
Queried gRNA [variant]:
GTAGGTCCATTGATGTTCAACCT
AGACTAAACATCAATGGACCAAC
||  | ||||||||||||||| ||
AGGTTGAACATCAATGGACCTAC
MD:Z:2GT1G14T2 NM:i:4

ID:3Dg2 Variant:AY053402.1 Pos:6094
Original gRNA [EV-A71]:
TTGGAAAACAGGGCTTGTTCAAA
Queried gRNA [variant]:
TTTGAAAATAAAGCCTGCTCAAA
TTTGAACAAGCCCTGTTTTCCAA
||||| || ||  | ||||| ||
TTTGAGCAGGCTTTATTTTCAAA
MD:Z:5G2G2TT1A5A2 NM:i:6

ID:3Dg3 Variant:AY053402.1 Pos:6217
Original gRNA [EV-A71]:
TAGCACGCTTCCTCCATGCTCAT
Queried gRNA [variant]:
TAACAAGCATCCTCCATGCTCAT
ATGAGCATGGAGGAAGCGTGCTA
|||||||||||||| || || ||
ATGAGCATGGAGGATGCTTGTTA
MD:Z:14T2T2T2 NM:i:3

ID:3Dg4 Variant:AY053402.1 Pos:6355
Original gRNA [EV-A71]:
TATTTATCCATGTAGAATTTCAT
Queried gRNA [variant]:
TACTTGTCCATGTAGAACTTCAT
ATGAAATTCTACATGGATAAATA
||||| |||||||||||| || ||
ATGAAGTTCTACATGGACAAGTA
MD:Z:5G11C2G2 NM:i:3

ID:3Dg5 Variant:AY053402.1 Pos:6826
Original gRNA [EV-A71]:
ATGATGTTGTTGATCATTGAATT
Queried gRNA [variant]:
ATGATATTATTGATCATCGAGTT
AATTCAATGATCAACAACATCAT
|| || |||||||| || |||||
AACTCGATGATCAATAATATCAT
MD:Z:2C2G8T2T5 NM:i:4

ID:3Dg6 Variant:AY053402.1 Pos:6988
Original gRNA [EV-A71]:
TCTGCAGGAGTCATGGTCAAACC
Queried gRNA [variant]:
TCGGCAGGGGTCATAGTCAAACC
GGTTTGACCATGACTCCTGCAGA
|||||||| ||||| ||||| ||
GGTTTGACTATGACCCCTGCCGA
MD:Z:8T5C5C2 NM:i:3
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B5 strain (FJ461781.1)

```
ID:3Dg1 Variant:FJ461781.1 Pos:5942
Original gRNA [EV-A71]:
GTTGGTCCATTGATGTTTAGTCT
Queried gRNA [variant]:
GTTGGTCCATTGATGTTCAGTCT
AGACTAAACATCAATGGACCAAC
||||| ||||||||||||||||||
AGACTGAACATCAATGGACCAAC
MD:Z:5G17 NM:i:1

ID:3Dg2 Variant:FJ461781.1 Pos:6062
Original gRNA [EV-A71]:
TTGGAAAACAGGGCTTGTTCAAA
Queried gRNA [variant]:
TTGGAAAACAGAGCCTGCTCAAA
TTTGAACAAGCCCTGTTTTCCAA
||||| || || ||||||||||
TTTGAGCAGGCTCTGTTTTCCAA
MD:Z:5G2G2T11 NM:i:3

ID:3Dg3 Variant:FJ461781.1 Pos:6185
Original gRNA [EV-A71]:
TAGCACGCTTCCTCCATGCTCAT
Queried gRNA [variant]:
TAGCACGCCTCCTCCATGCTCAT
ATGAGCATGGAGGAAGCGTGCTA
|||||||||||||| ||||||||
ATGAGCATGGAGGAGGCGTGCTA
MD:Z:14G8 NM:i:1

ID:3Dg4 Variant:FJ461781.1 Pos:6323
Original gRNA [EV-A71]:
TATTTATCCATGTAGAATTTCAT
Queried gRNA [variant]:
TATTTATCCATATAAAATTTCAT
ATGAAATTCTACATGGATAAATA
|||||||| || ||||||||||
ATGAAATTTTATATGGATAAATA
MD:Z:8T2T11 NM:i:2

ID:3Dg5 Variant:FJ461781.1 Pos:6794
Original gRNA [EV-A71]:
ATGATGTTGTTGATCATTGAATT
Queried gRNA [variant]:
ATGATGTTGTTGATCATTGAATT
AATTCAATGATCAACAACATCAT
||||||||||||||||||||||
AATTCAATGATCAACAACATCAT
MD:Z:23 NM:i:0

ID:3Dg6 Variant:FJ461781.1 Pos:6956
Original gRNA [EV-A71]:
TCTGCAGGAGTCATGGTCAAACC
Queried gRNA [variant]:
TCTGCAGGTGTCATAGTCAAACC
GGTTTGACCATGACTCCTGCAGA
|||||||| ||||| ||||||||
GGTTTGACTATGACACCTGCAGA
MD:Z:8T5A8 NM:i:2
```

C4 strain (JQ965759.1)

```
ID:3Dg1 Variant:JQ965759.1 Pos:5973
Original gRNA [EV-A71]: GTTGGTCCATTGATGTTTAGTCT
Queried gRNA [variant]: GTTGGTCCATTGATGTTGAGTCT
AGACTAAACATCAATGGACCAAC
||||| ||||||||||||||||
AGACTCAACATCAATGGACCAAC
MD:Z:5C17 NM:i:1

ID:3Dg2 Variant:JQ965759.1 Pos:6093
Original gRNA [EV-A71]: TTGGAAAACAGGGCTTGTTCAAA
Queried gRNA [variant]: TTAGAGAACAGGGCATGCTCAAA
TTTGAACAAGCCCTGTTTTCCAA
||||| || |||||||| || ||
TTTGAGCATGCCCTGTTCTCTAA
MD:Z:5G2T8C2T2 NM:i:4

ID:3Dg3 Variant:JQ965759.1 Pos:6216
Original gRNA [EV-A71]: TAGCACGCTTCCTCCATGCTCAT
Queried gRNA [variant]: TAGCAGGCCTCCTCCATGCTCAT
ATGAGCATGGAGGAAGCGTGCTA
|||||||||||||| || |||||
ATGAGCATGGAGGAGGCCTGCTA
MD:Z:14G2C5 NM:i:2

ID:3Dg4 Variant:JQ965759.1 Pos:6354
Original gRNA [EV-A71]: TATTTATCCATGTAGAATTTCAT
Queried gRNA [variant]: TATTTGTCCATGTAGAACTTCAT
ATGAAATTCTACATGGATAAATA
||||| |||||||||||| |||||
ATGAAGTTCTACATGGACAAATA
MD:Z:5G11C5 NM:i:2

ID:3Dg5 Variant:JQ965759.1 Pos:6825
Original gRNA [EV-A71]: ATGATGTTGTTGATCATTGAATT
Queried gRNA [variant]: ATAATGTTGTTGATCATTGAGTT
AATTCAATGATCAACAACATCAT
|| ||||||||||||||||| ||
AACTCAATGATCAACAACATTAT
MD:Z:2C17T2 NM:i:2

ID:3Dg6 Variant:JQ965759.1 Pos:6987
Original gRNA [EV-A71]: TCTGCAGGAGTCATGGTCAAACC
Queried gRNA [variant]: TCTGCAGGAGTCATGGTTAGGCC
GGTTTGACCATGACTCCTGCAGA
||  | ||||||||||||||||||
GGCCTAACCATGACTCCTGCAGA
MD:Z:2CC1A17 NM:i:3
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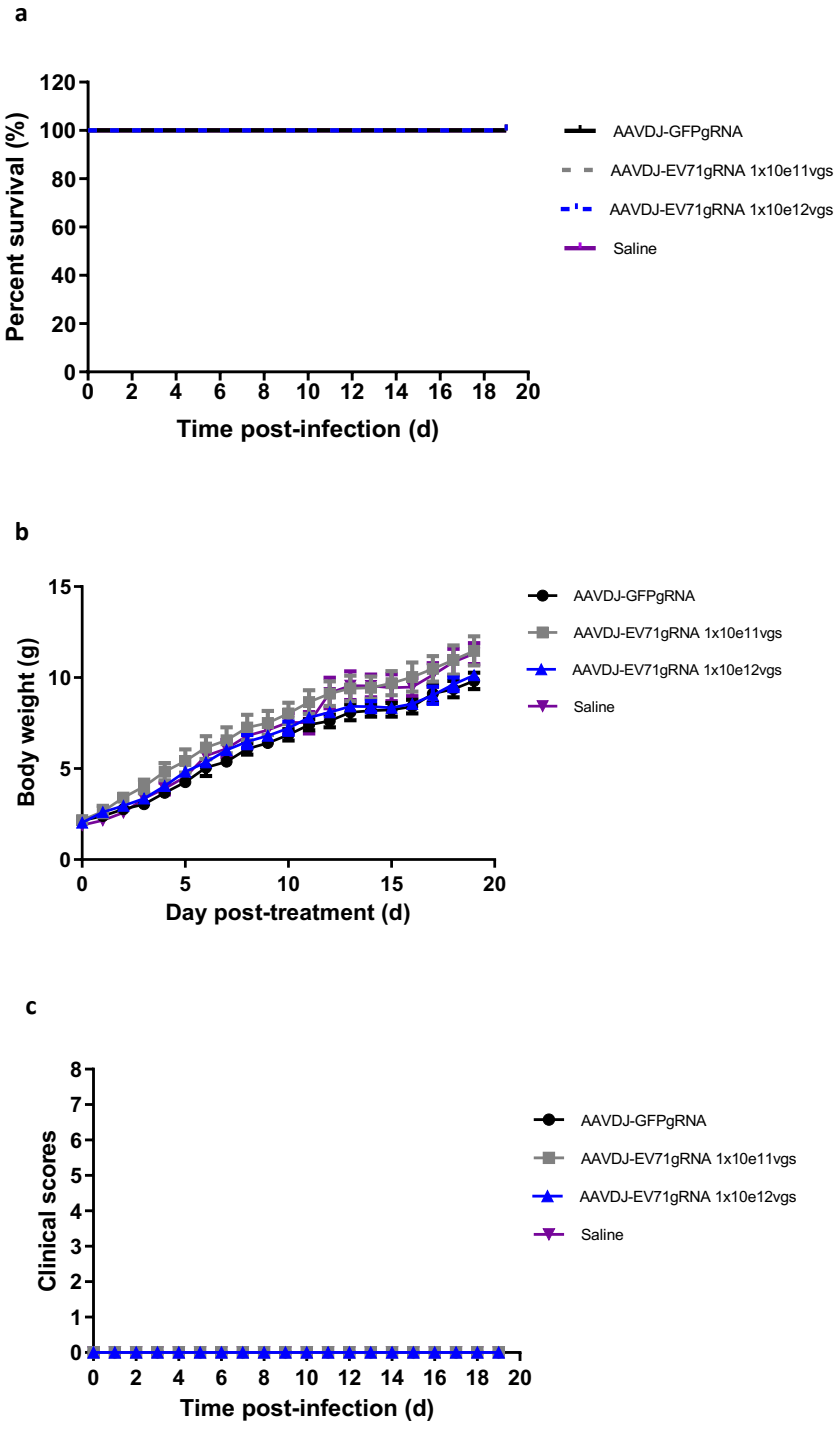
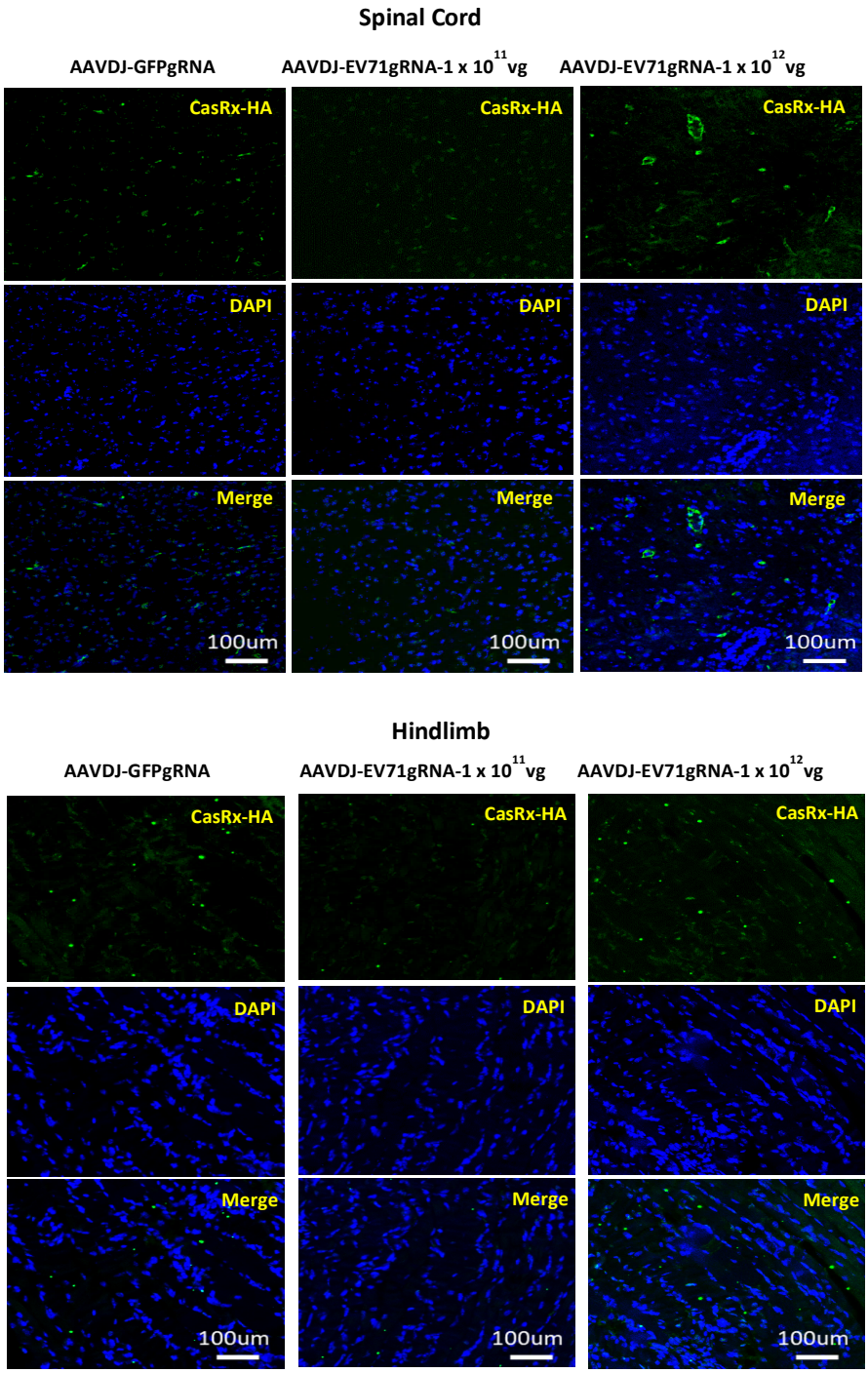
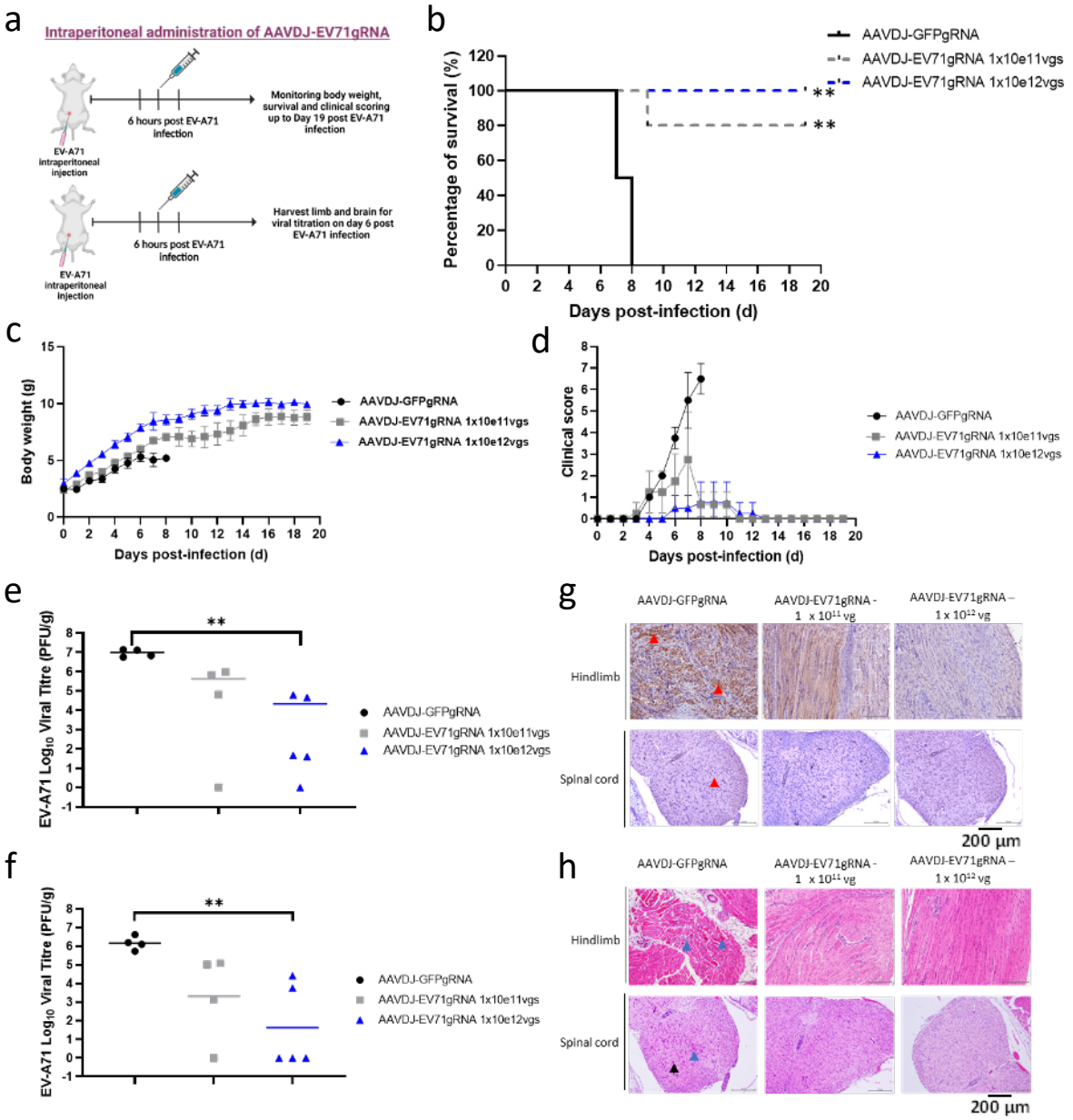
Supplementary Figure 6. Alignment of gRNAs targeting HFM41 lab strain against different EV-A71 strains.



Supplementary Figure 7. AAVDJ-CRISPR-CasRx does not induce overt toxicity at the treatment dosages. a. BALB/c mice were injected intraperitoneally with saline, 1 x 10^{12} vgs AAVDJ-GFPgRNA, 1 x 10^{11} vgs of AAVDJ-EV71gRNAs, or 1 x 10^{12} vgs of AAVDJ-EV71gRNAs per 2-day old mouse. Survival was recorded for 19 days post-infection (dpi). **b**. The body weight of each mouse in each treatment group was recorded for 19 dpi. **c**. The clinical score of mice was recorded using the mice clinical assessment scoring system (M-CASS) involving observation of five markers: activity, breathing, movement, body weight, and dehydration for up to 19 dpi.



Supplementary Figure 8. Expression of CasRx-HA in mice transduced with AAVDJ-CasRx-EV71gRNA. Tissue sections were stained with anti-HA antibodies and counterstained with DAPI. CasRx-HA staining was observed in all groups for both spinal cord and hind limb tissues (top row). Stronger CasRx staining was observed in tissues of mice transduced with 1 x 10^{12} vgs of AAVDJ-GFPgRNA (left column) and 1 x 10^{12} vgs of AAVDJ-EV71gRNA (right column) compared to tissues of mice transduced with 1 x 10^{11} vgs AAVDJ-EV71gRNA (centre column). Magnification 20X; Scale bar, 100µm.



Supplementary Figure 9. AAVDJ-CRISPR-CasRx prevents mortality and reduces pathology when applied 6hrs after EV-A71 infection. a. BALB/c mice were injected intraperitoneally with a dose of 2×10^7 PFU EV-A71 per mouse at 5 days old. After 6 hours, mice were injected intraperitoneally with a dose of 1×10^{11} viral genomes (vgs) or 1×10^{12} vgs of AAVDJ-EV71gRNAs per mouse. 1×10^{12} vgs AAVDJ-GFPgRNA was used as treatment control. **b.** The survival of the mice was recorded for 19 days post-infection (dpi). Comparison between two groups (CasRx-GFPg2 and CasRx-EV71_3Dguides) was analyzed by Log-rank (Mantel-Cox) test. **p<0.005. **c.** The body weight of each mouse in each treatment group was recorded for 19 dpi. **d.** The clinical score of each mouse was recorded using the mice clinical assessment scoring system (M-CASS) for up to 19 dpi. **e.** Virus titers in the hind limbs of mice as determined using the plaque-forming assay. Virus titration results were from a single experiment. **f.** Virus titers in the brains of mice as determined using plaque-forming assay; Kruskal-Wallis test with Dunn's multiple comparisons post hoc test. **p<0.005. Virus titration

result were from a single experiment. **g.** Immuno-histochemistry staining for EV-A71 specific antigen (*red arrow*). **h.** H&E staining of the hind limbs and spinal cords of mice. Polymorphonuclear meningitis in the spinal cord (*black arrow*). Necrosis and focal interstitial mononuclear cell infiltrate in the hind limbs (*blue arrow*). Magnification of H&E and IHC images are taken at 200X.