High ionic strength vector formulations enhance gene transfer to airway epithelia

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One Sentence Summary: Formulating adenoviral, AAV, and lentiviral vectors with hypertonic saline remarkably enhances lung gene transfer. (114 characters, including spaces)

ABSTRACT A fundamental challenge for cystic fibrosis (CF) gene therapy is ensuring sufficient transduction of airway epithelia to achieve therapeutic correction. Hypertonic saline (HTS) is frequently administered to people with CF to enhance mucus clearance. HTS transiently disrupts epithelial cell tight junctions, but its ability to improve gene transfer has not been investigated. Here we asked if increasing the concentration of NaCl enhances the transduction efficiency of three gene therapy vectors: adenovirus, AAV, and lentiviral vectors. Vectors formulated with 3-7% NaCl exhibited markedly increased transduction for all three platforms, leading to anion channel correction in primary cultures of human CF epithelial cells and enhanced gene transfer in mouse and pig airways *in vivo*. The mechanism of transduction enhancement involved tonicity but not osmolarity or pH. Formulating vectors with a high ionic strength solution is a simple strategy to greatly enhance efficacy and immediately improve preclinical or clinical applications.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by pathogenic variants of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. CFTR protein conducts Cl^- and HCO_3^- anions at the apical surface of epithelial cells. Decreased activity or loss of CFTR in the airways leads to a series of complications including bacterial colonization, chronic inflammation, and mucus accumulation. Numerous studies have established that *CFTR* complementation restores anion transport both *in vitro* and *in vivo*[1-3]. The advent of the small molecule therapy of elexacaftor, tezacaftor, and ivacaftor (ETI), trade name Trikafta, demonstrated that restoring CFTR improves lung function and quality of life in people with CF. Although ETI treatment benefits the majority of people with CF, there remains a pressing need to develop treatments for all people with CF. Gene therapy by *CFTR* complementation can restore anion channel activity regardless of the mutation. Despite numerous clinical trials (reviewed in[4]), no CF gene therapies have advanced past Phase II trials due, in part, to low efficacy of CFTR gene transfer.

The conducting airways are challenging to transduce using topically applied gene therapy reagents due to an enormous surface area and multiple host defense mechanisms to resist viral vector uptake[4]. Vehicle formulations such as lysophosphatidylcholine (LPC)[2, 5], EGTA[6, 7], methylcellulose[8], and perflurocarbon[9] improve viral vector transduction and subsequent transgene expression in cultured cells and animal models; however, none of these examples are currently FDA approved for pulmonary administration. The ideal vector formulation that enhances gene transfer would be FDA approved and cost effective. Hypertonic saline (HTS) is commonly aerosolized at 3-7% as a topical lung treatment for people with CF to improve mucus clearance. HTS transiently disrupts tight junctions[10] and exhibits multiple actions including: a mucolytic agent (disrupting mucus gel), an expectorant (hydrolyzing airway surface liquid), a mucokinetic agent

(promoting cough-mediated clearance), and increasing ion flux of two thiols (glutathione and thiocyanate) that protect against oxidative injury (reviewed in[11]). Thus, HTS hydrates surface epithelia, breaks ionic bonds, dislodges mucus, and enhances mucociliary clearance[12]. Clinical benefits and safety track records of this treatment are well established for people with CF.

Here we screened reagents approved for topical airway delivery. We were interested in reagents which decreased transepithelial resistance to increase access to basolateral receptors and enhance gene transfer. We formulated adenoviral (Ad) vectors, adeno-associated viral (AAV) vectors, or the lentiviral vector human immunideficiency virus (HIV) with 1-7% NaCl and applied the mixtures to the apical surface of primary human airway epithelial cells (HAE). We found that increasing the NaCl tonicity of the vector formulation correlated with enhanced gene transfer in a dose dependent fashion and functionally restored CFTR anion transport *in vitro*. Focused entry studies with Ad suggest that vector transduction improves with increasing ionic strength, remains receptor-mediated, and requires a low pH endosomal step. Finally, we asked if vectors delivered in hypertonic saline enhanced gene transfer *ex vivo* and *in vivo*. Ad and AAV transduction was significantly increased in pig explants *ex vivo*. When we delivered Ad with NaCl to both mouse (7% NaCl) and pig airways (5% NaCl), we observed significantly enhanced gene transfer *in vivo*. Additionally, we aerosolized GP64 pseudotyped HIV lentivirus formulated in 5% NaCl to pig airways and observed widespread reporter gene expression greater than the isotonic saline control. Together, these findings indicate that formulating viral vectors with hypertonic saline for gene addition or gene editing approaches may be a simple means to improve transduction.

RESULTS

Not all vehicles that decrease transepithelial resistance enhance gene transfer

Disrupting the epithelial cell tight junctions allows Ad5-based viral vectors access to their basolaterally localized coxsackie and adenovirus receptor (CXADR)[13]. Unless otherwise specified, these studies use the Ad5 serotype. We screened several compounds for their ability to reduce the transepithelial electrical resistance (TEER) of HAE and improve transduction of an Ad vector. First, reagents were applied to the apical surface of HAE for 5 minutes and then TEER was measured using an ohmmeter. Consistent with previous reports, lysophosphatidylcholine (LPC) (0.1%) and EGTA (250 mM) reduced TEER and increased gene transfer[2, 6]. The clinically approved reagents doxorubicin (5 µM), NaCl (3.6%), and surfactant (Infasurf, 35 mg/ml) all decreased TEER (**Supplemental Fig. 1A**). In separate cultures, Ad-GFP (MOI=250) in 50 µl of DMEM combined 1:1 with the test reagents was applied to the apical surface for 2 hours. 5 days post-treatment, cells were imaged by fluorescence microscopy. Doxorubicin, surfactant, and 0.9% NaCl (PBS)

did not enhance Ad gene transfer. Of note, the 3.6% NaCl formulation (mixed 1:1 from 7.2%) conferred remarkable transduction (**Supplemental Fig. 1B**). Based on this initial observation, this concentration of NaCl was selected for the many of the subsequently described Ad-based studies.

Increasing saline tonicity enhances gene transfer in a dose and time dependent manner

To determine if increasing saline tonicity further improves Ad transduction in HAE, we performed a dose-response of 1-7% NaCl formulation (final concentration, **Table 1**) with Ad-GFP (MOI=250). We observed a dose-dependent increase in transduction efficiency with increasing NaCl tonicity (**Fig. 1A, B**). Basal, secretory, and ciliated cells were all transduced in a dose-dependent manner (**Fig. 1C, Supplemental Fig. 2A**). HAE permeability to dextran also increased in the presence of 4-7% NaCl (**Fig. 1D**), which correlated with increasing GFP expression (**Fig. 1E**). The highest concentrations of NaCl (5-7%) reduced cell viability as determined by flow cytometry (**Supplemental Fig. 2B**) and increased LDH release (**Supplemental Fig. 2C**). To visualize that cell surface integrity and ciliation remained intact, we performed scanning electron microscopy (SEM) on HAE that were untreated or treated with NaCl (3.6%) for 2 hours and observed no overt physical differences compared to the no NaCl control (**Supplemental Fig. 2D**). The therapeutic range of NaCl delivered to patients by aerosolization is up to 7%[14]; however, based on the cell viabilty results, we restricted the delivered concentration of NaCl to 3.5-4.5% for subsequent *in vitro* studies.

We next asked how the length of exposure to NaCl or Ad impacted transduction in cultured HAE. As depicted schematically (**Supplemental Fig. 3A**), HAE were incubated with constant Ad-GFP for 120 min and varied times for NaCl (3.6%) at 5, 15, 30, 60, or 120 min. In parallel, HAE were incubated with constant NaCl (3.6%) and varied Ad-GFP incubation times. GFP expression was visualized 5 days post-transduction and quantified by flow cytometry (**Supplemental Fig. 3B**). The %GFP+ cells increased incrementally with increasing temporal exposure to either Ad-GFP or NaCl, with significant increase at 1 hour Ad-GFP with constant NaCl (3.6%) exposure and maximal expression after a 2 hour co-treatment. Given these time-dependent results, we established a standard 2 hour co-delivery protocol.

NaCl-mediated Ad transduction restores anion transport

We hypothesized that the NaCl conferred increase in transduction efficiency would enhance Ad-CFTR complementation of anion transport in CF airway epithelia. We next co-delivered Ad-CFTR formulated with NaCl (3.6%) to the apical surface of primary CF HAE for 2 hours. Five days post-delivery, bioelectric properties were measured in Ussing chambers as previously reported[15]. Without NaCl (3.6%) formulation,

no change in Cl^- conductance was observed in response to the CFTR agonists forskolin and IBMX (F&I) or CFTR inhibitor GlyH in cultured primary CF epithelia treated with Ad-CFTR (**Fig. 2C**). In contrast, cells that received Ad-CFTR and NaCl (3.6%) showed wild-type levels of Cl^- secretion as measured by short circuit current (**Fig. 2A, B**) and conductance (**Fig. 2C, D**). Parallel CF airway cultures were transduced as indicated to determine transgene expression by microscopy (**Fig. 2E**) and measured by flow cytometry (**Fig. 2F**) or western blot (**Fig. 2F, inset**). These results suggest that NaCl formulation of Ad-CFTR improved gene transfer and conferred wild-type levels of CFTR mediated anion transport in CF epithelial cells.

Impact of osmolarity, pH, and ionic strength on NaCl enhanced gene transfer

To investigate possible mechanisms for the enhanced transduction by NaCl formulations, we compared 3.6% NaCl to the ionic osmolytes 3.6% $NaC_6H_{11}O_7$ (NaGluconate), 3.6% KCl, 0.9% NaCl, and 0.9% NaCl with the non-ionic osmolyte mannitol of equivalent osmolarity to the NaCl (3.6%) formulation (**Table 2**). The indicated regents were co-delivered with Ad-GFP apically to HAE for two hours. Microscopy and flow cytometry quantification was performed five days later. NaCl (3.6%) conferred the greatest increase in transduction efficiency (**Fig. 3A, B**), whereas equivalent tonicity (3.6%) or osmolarity (620 mM, 1,230 mOsmo/L) with ionic or non-ionic osmolytes did not significantly enhance gene transfer. We next determined if the pH of NaCl formulation impacts transduction. Given that the unadjusted pH of 3.6% NaCl in deionized water is ~6, we adjusted the pH of the NaCl (3.6%) solution to 5, 6, 7, 7.4, and 8. Following Ad-GFP delivery, and flow cytometry quantification protocols in HAE, no significant differences in %GFP+ cells were observed across this pH range (**Fig. 3C**). To investigate another potential mechansim, we asked if ionic strength was responsible for enhanced vector transduction. We compared KCl (lower ionic strength than NaCl for a given percent solution), mannitol (uncharged sugar) and N-methyl-D-glucamine gluconate (charged sugars) at 1-7% tonicities (**Table 1** and **Fig. 3D**). We observed dose-dependent increases with KCl and N-methyl-D-glucamine gluconate whereas mannitol did not enhance gene transfer (**Fig. 3E-G**). The formulation was not dependent on osmolarity or pH, but ionic strength increased the Ad transduction efficiency.

NaCl-mediated gene transfer remains receptor dependent and requires a low pH endosome

Next, we investigated whether the NaCl enhanced Ad5 transduction is receptor dependent, using CRISPR/Cas9 RNPs to knockout the Ad5 receptor CXADR in primary human airway basal cells[16-18]. The electroporated primary cells were seeded on transwell membranes and cultured at an air-liquid interface until well-differentiated (~21 days). $CXADR^{KO}$ cells and donor-matched airway epithelia were transduced with

Ad5-GFP and Ad21-GFP in the presence or absence of NaCl (3.6%). Ad21 enters airway cells through a CXADR-independent mechanism using CD46 as a cellular receptor[19]. Ad5 + NaCl did not transduce $CXADR^{KO}$ epithelia, whereas Ad21 + NaCl readily transduced $CXADR^{KO}$ cells (**Fig. 4A, B**). Following receptor engagement and internalization, Ad5 transduction requires escape from low pH endosomes. Inhibiting endosomal acidification with chloroquine (CQ) or bafilomycin A1 (Baf A1) significantly decreased the NaCl enhanced transduction (**Fig. 4C**). These results indicate NaCl enhanced transduction requires receptor engagement and low pH endosomal release.

NaCl formulations enhance lentiviral and AAV gene transfer

We next asked if formulating lentiviral or adeno-associated viral (AAV) vectors with increasing concentrations of NaCl enhanced transduction. An HIV-based viral vector pseudotyped with the baculoviral GP64 envelope glycoprotein[20] was applied to the apical surface of HAE with 3.5, 4, or 4.5% NaCl. A dose-dependent increase in GFP expression was observed in non-CF HAE (**Fig. 5A**) and we therefore selected 4.5% NaCl for AAV and lentiviral studies. Primary human CF cultures that received GP64 HIV-GFP (MOI=50) also showed remarkable levels of transduction (**Fig. 5B**). Notably, in the presence of NaCl (4.5%), GP64 HIV-CFTR restored the CFTR-dependent anion transport in primary CF HAE. Without NaCl formulation, improvements in CFTR-dependent currents were not observed (**Fig. 5C, D**). Interestingly, NaCl-mediated entry did not enhance transduction of all pseudotyped lentiviral vectors. A screen of additional envelopes from Vesicular Stomatitis Virus (VSVG)[21], Jaagsiekte Sheep Retrovirus (JSRV)[22], and Baboon Endogenous Virus (BaEV)[23] in the presence or absence of NaCl (4.5%) revealed that only GP64 and VSVG showed enhanced transduction (**Supplemental Fig. 4**).

To test the effect of NaCl formulation on AAV-based vectors, we selected the AAV2.5T capsid based on previously established airway tropism[24]. Similar to Ad and lentiviral studies, we applied AAV-GFP (MOI=100,000 vg/cell) to the apical surface of HAE for 2 hours with or without NaCl (4.5%). Doxorubicin (1 μM) was added to the basolateral media 24 hours prior to the 2 hour AAV application. A 2 hour application was chosen to be consistent with the Ad and HIV protocols, however we and others have shown that overnight applications of AAV are effective in the absence of NaCl[25]. Microscopy, flow cytometry quantification, and Ussing chamber assays were performed 2 weeks post-transduction. The epithelia treated with NaCl (4.5%) formulated AAV-GFP achieved significantly higher GFP expression (**Fig. 5E**) and increased anion channel activity in responses to F&I and GlyH (**Fig. 5F, G**). These results confirm that transduction with NaCl formulation is enhanced across all three viral vector systems.

Increased saline tonicity enhances gene transfer *ex vivo* and *in vivo*

We next asked if formulating vectors in NaCl (5%) would enhance gene transfer in an *ex vivo* model. Using freshly excised newborn pig tracheal explants maintained on Surgifoam, we applied Ad-GFP or AAVH22-GFP (a pig-tropic AAV capsid)[26] ± NaCl (5%) to the apical surface for 2 hours. One week later, tissues were fixed and imaged by confocal microscopy. Fluorescence was quantified by transduction area per high power field (**Supplemental Fig. 5A, B**). Transduction with both Ad and AAVH22 was markedly enhanced in *ex vivo* tracheal explants when formulated with NaCl (5%).

Improving viral vector transduction *in vivo* has broad implications for advancing gene therapy for lung diseases. We first asked if vectors formulated with NaCl (7%) improved gene transfer to mouse lungs. Ad-luciferase was formulated with NaCl (7%) or NaCl (0.9%) and delivered intratracheally. 5 days post-delivery, animals treated with vectors formulated in 7% NaCl demonstrated significantly increased luciferase expression relative to saline control mice (**Fig. 6A**). We next asked whether NaCl formulation would improve gene transfer in a large animal model. Newborn pigs received Ad-GFP via intratracheal delivery with PBS or NaCl (5%). Five days after delivery, lungs were collected and divided into 20 regions for systematic quantification as previously described[2] (**Fig. 6B**). GFP expression was scored using a fluorescence dissecting microscope (0 = no expression, 1 = low expression, 2 = moderate expression, and 3 = high expression). Formulation of Ad-GFP with NaCl (5%) resulted in a significantly increased the GFP+ score compared to PBS (**Fig. 6C-E**). **Fig. 6F** shows representative images of NaCl (5%) treated lung sections at 2x and 10x, respectively. We also tested GP64 HIV-GFP formulated with NaCl (5%) or PBS in pigs. We observed significantly enhanced transduction using NaCl formulation over PBS (**Fig. 6G-I**). In summary, formulating either Ad or lentiviral vectors in 5-7% NaCl enhanced airway and alveolar gene transfer in mice and pigs.

DISCUSSION

Here we show that NaCl (3.6-7%) enhanced the transduction efficiency of Ad, AAV, and lentiviral vectors. This improved transduction restored functional CFTR anion transport in CF airway epithelia across all 3 vector platforms. For adenovirus vectors, the NaCl formulation enhanced transduction remained receptor dependent, requiring a low pH endosomal escape. These *in vitro* findings of enhanced transduction also translated to two animal models where formulating Ad or lentiviral vectors with 5% or 7% NaCl increased transgene expression. Given that 3-7% NaCl is routinely aerosolized in people with CF[27], these findings have

important implications for improving viral vector transduction for CF and other lung diseases. Formulating viral vectors with 3.6-7% NaCl could boost therapeutic efficacy, lower the therapeutic index, and overcome previous expression threshold limitations for pulmonary gene therapy.

Formulations including LPC[5], EGTA[6, 7], perfluorocarbon[9], and methylcellulose[8] have been instrumental in *in vitro* and pre-clinical *in vivo* studies to improve gene transfer efficiency. Interestingly, the discovery that Ad-based viral vectors use a basolateral receptor and gene transfer is boosted by tight junction disruption came to light after the first CF gene therapy clinical trials[4]. Since none of the above mentioned formulations are currently FDA approved for lung administration, each reagent would require a separate evaluation for co-administration with a gene therapy vector. CF gene therapy clinical trials performed to date include vehicles with a 150 mM NaCl concentration, which is equivalent to 0.9% isotonic NaCl (reviewed in[4]). Viral vectors including AAV and lentiviral platforms are under investigation for CF gene therapy applications. 4D Molecular Therapeutics began a Phase I AAV clinical trial in 2022 (clinicaltrials.gov) and Spirovant and Carbon Biosciences are generating preclinical data to pursue clinical trials with AAV and bocavirus vectors. Historically, achieving sufficient gene transfer for phenotypic correction has been challenging[4]. Efforts to overcome these challenges include directed evolution for capsid[24] and envelope design[20], cassette modification including promoter[28], cDNA[29], and polyadenylation signal, codon optimization[21], and immunomodulation (reviewed in[4]). Using a formulation generally recognized as safe for use in people with CF such as NaCl (3-7%) could eliminate a hurdle for approval and advancement of a gene therapy reagent.

The effects of increased saline tonicity formulation on viral vector transduction have not been previously investigated. To our knowledge, only one relavant report suggests that increased saline tonicity does not enhance SARS-CoV-2 viral entry[30]. In screening several lentiviral envelope pseudotypes (Supplemental Fig. 4) we observed that vectors with the GP64 and VSVG envelopes benefited from NaCl (4.5%) formulation, while JSRV or BaEV did not. JSRV uses the GPI-linked apical receptor Hyal2[31] while the BaEV uses SLC1A4 and SLC1A5 as receptors. Although these receptors are present in HAE, transduction was not enhanced with NaCl formulation. These results suggest that entry pathways may influence the effectiveness of NaCl formulations. Indeed, different viruses have differing entry routes, including receptor-mediated endocytosis or fusion at the plasma membrane. Further, it may be that some receptors, co-receptors, and other binding or entry factors may be affected by the presence of high NaCl while others are not. We speculate that virus like particles (VLPs)[32] pseudotyped with envelopes such as VSVG or GP64 would also be enhanced when formulated with hypertonic saline.

We originally suspected the mechanism for NaCl-mediated enhanced transduction was due to the osmotic strength of the formulation. We know that the airway surface liquid volume is regulated in part by water movement through the cell and that osmotically driven water permeability is regulated by aquaporins to maintain airway epithelial cell homeostasis[33, 34]. When a hypertonic solution is applied to epithelia, an electrochemical gradient generates a driving force for fluid movement across the cell membrane, causing hypertonic shock, followed by an adaptive regulatory increase in cell volume. The non-ionic osmolyte mannitol did not enhance transduction. Instead, we learned that ionic strength is a key determinant in the mechanism by which NaCl formulation enhance vector transduction. We speculate that NaCl formulation may enhance transduction, in part, by increasing receptor access by disrupting tight junctions or by enhancing endosomal escape. We observed that NaCl, KCl, and the charged sugar N-methyl-D-glucamine enhanced transduction in a dose-dependent manner, although NaCl had the greatest impact in a given percent solution. These findings suggest that Na^+ regulation of the airway extracellular environment has the greatest impact on viral transduction[35].

As mentioned in the Results, a concentration of 3.6% final concentration of NaCl was serendipitously chosen for most of the studies using Ad because it was initially diluted 1:1 from a 7.2% pharmaceutical grade stock solution. For *in vitro* studies in HAE, our dose response results suggest that 4.5% NaCl may be the best compromise between efficacy and ultimate toxicity at doses of NaCl >6%. This was true for all vectors tested. Of note, attempts to improve gene transfer of cultured cells on plastic using hypertonic saline resulted in lifting of the cells. Our pilot studies *in vivo* suggested that vector formulations of 5-7% NaCl had the greatest impact on gene transfer to the lungs of mice and pigs. This suggests that the dose limitation *in vitro* may not apply to *in vivo* gene transfer and higher initial concentrations of NaCl may be necessary to counteract the immediate dilution of the encountered airway surface liquid.

The goal of this study was to investigate whether FDA approved agents could improve lung gene therapy efficacy. Hypertonic saline is a routinely used therapy for people with CF and other lung conditions and could be rapidly adopted in vector formulations. We show evidence that formulating Ad, AAV or lentiviral vectors with NaCl could advance gene therapy development for CF. Importantly, this finding has broad implications for other genetic lung diseases such as alveolar type 2 deficiencies (ABCA3 Deficiency, Surfactant Protein B Deficiency) or Primary Ciliary Dyskenesia.

MATERIALS AND METHODS

Ethics statement

Primary airway epithelia from human CF and non-CF donors were isolated from discarded tissue, autopsy, or surgical specimens. Cells were provided by The University of Iowa *In Vitro* Models and Cell Culture Core Repository. Information that could be used to identify a subject was not provided. All studies involving human subjects received University of Iowa Institutional Review Board approval (Protocol #230167). Mice and pig experimental protocols were reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee (IACUC), in accordance with the United States Department of Agriculture and National Institutes of Health guidelines.

Human airway epithelial cells

The University of Iowa *In Vitro* Models and Cell Culture Core cultured and maintained HAE as previously described[36]. Briefly, following enzymatic disassociation of trachea and bronchus epithelia, the cells were seeded onto collagen-coated, polycarbonate Transwell inserts (0.4 μm pore size; surface area = 0.33 cm^2; Corning Costar, Cambridge, MA). HAE were submerged in Ultroser G (USG) medium for 24 hours (37°C and 5% CO_2) at which point the apical media is removed to encourage polarization and differentiation at an air-liquid interface. Transepithelial electrical resistance was measured using an Ohmmeter ($\Omega \cdot \mu m^2$).

Viral vector production and formulation

Vectors were produced by the University of Iowa Viral Vector Core (https://medicine.uiowa.edu/vectorcore/). Ad5 and Ad21 CMV-eGFP CMV-mCherry, or F5Tg83-CFTR was produce, purified, and titered as previously described[37]. AAV2/2.5T, AAV2/H22 CMV-eGFP or F5Tg83-CFTRΔR was produced by triple transfection. Lentiviral HIV-CMV-eGFP or PGK-CFTR vectors pseudotyped with GP64 were produced by a four-plasmid transfection method as previously described[38, 39] and titered using droplet digital PCR [40] and/or by flow cytometry. Similarly, VSVG, JSRV and BaEV pseudotyped lentiviral vectors were made in-house by four-plasmid transfection and titered by flow cytometry. Viral vectors were formulated with indicated NaCl concentrations (presented as final concentrations). The following MOIs were used for each vector: Ad=250, AAV=100,000, and HIV=50. Ad studies were performed by mixing pharmaceutical grade 7.2% NaCl with Ad-GFP (3.6% final). Additional concentrations were made from a 10% NaCl solution of Sodium Chloride (Research Products International, Mount Prospect, IL) in UltraPure Distilled Water (Invitrogen, Waltham, MA). Vectors were formulated with NaCl in a final volume of 50 μl and applied to the apical surface of HAE for 2 hours. AAV transduced cultures were pre-treated with indicated concentraions of doxorubicin [1 μM] for 24 hours prior to 2 hour vector application. Other formulations tested include lysophosphatidylcholine (LPC) (9008-30-4, Millipore

Sigma, St. Louis, MO), Ethylene glycol-bis-2-aminoethylether)-N,N,N'N'-tetraacetic acid (EGTA) (Research Products International, Mount Prospect, IL), and surfactant (Infasurf, pharmaceutical grade).

Fluorescence microscopy and flow cytometry

GFP images were acquired using a Keyence All-in-one Fluorescence Microscope BZ-X series (Osaka, Japan). 0.33 cm^2 transwells were imaged at 2X magnification. GFP expression was quantified by flow cytometry as previously reported[39, 41]. Briefly, cells were stained with a fixable LIVE/DEAD stain (Thermo Fisher Scientific, Waltham, MA), lifted in Accutase at 37°C for 30 minutes, and run through an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA). Cells were treated with the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's recommendations and stained for 1 hour at 4°C with the following antibodies: NGFR (345110; 1:600, BioLegend, San Diego, CA, USA), α-tubulin (NB100-69AF405, 1:300, Novus, Centennial, CO, USA) and CD66c (12-0667-42, 1:600, Invitrogen, Waltham, MA). Expression was gated on live cells.

LDH release assay

LDH release was quantified according to the manufacturer's recommendations (LDH-Glo Cytotoxicity Assay, Promega, Madison, WI). Briefly, basolateral media from each condition was collected in a 96 well plate. The LDH detection reagents were mixed in a 1:1 ratio and applied to each well and incubated for 30 minutes. Luminescence was recorded using a SpectraMax i3x plate reader.

Dextran permeability assay

Tight junction permeability was assessed by passage of 3000-5000 average molecular weight Fluorescein isothiocyanate-dextran (FD4, Sigma-Aldrich, St. Louis, MO) from the apical to basolateral side of well-differentiated human airway epithelia. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, St. Louis, MO) serves as a positive control for membrane permeabilization and was pre-treated (50 µM) overnight prior to addition of dextran. 250 mM EGTA pre-treatment for 2 hours was used for a positive experimental tight junction disruption control. Cells were left untreated or pre-treated with NaCl ranging from 1-7% for 2 hours. At the time of the assay, pre-treatments were removed and fresh basolateral media was added. 100 µl of 1 mg/ml dextran was applied apically for 30 minutes at 37°C. Basolateral media was assessed for fluorescent units using a SpectraMax i3x plate reader.

SEM

Scanning electron microscopy (SEM) samples were processed by the Johns Hopkins Institute for Biomedical Sciences Microscope Facility as a fee for service (https://microscopy.jhmi.edu/Services/EMCostStruct.html).

356

357 *Electrophysiology*

358 Short circuit current (I_{sc}) and conductance (G_{sc}) was measured as previously described[39]. Briefly, cells were
359 pre-stimulated with forskolin and IBMX (F&I) overnight and their bioelectric properties were quantified by
360 Ussing chamber analysis. Assay protocol is as follows: amiloride, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic
361 acid (DIDS), F&I, and GlyH-101 (GlyH). Results are reported as change in short circuit current (ΔI_{sc}) and
362 conductance (ΔG_{sc}) in response to F&I or GlyH.

363

364 *Western blot*

365 Protein from human airway epithelia transduced with Ad-CFTR alone or Ad-CFTR+NaCl (3.6%) was
366 harvested using RIPA buffer. 20 µg of each sample was loaded on a Criterion Tris-Glycine 4-20% gel and
367 run at 110V for 1 hour and transferred overnight onto a PVDF membrane at 110 µA at 4°C. After membrane
368 blocking, each membrane was probed for using the following primary antibodies: mouse anti-CFTR 596
369 (1:1000) (UNC) or CAR anti-rabbit (CXADR) (1:100) (PA5-31175, Invitrogen, Waltham, MA) was applied
370 for 2 hours. Following TBST washes, the LiCor secondary antibody was used at 1:10,000 for 30 minutes.
371 The membrane was imaged using an Odyssey imager.

372

373 *Sugar tonicity and ionic vs. non-ionic osmolytes*

374 KCl, mannitol, and N-methyl-D-glucamine (NMDG) gluconate were formulated 1-7% and co-delivered with
375 Ad-GFP (MOI=250) for 2 hours apically. Molarities and osmolarities are presented in Table 1. Sodium
376 chloride (NaCl), mannitol, sodium gluconate, and potassium chloride (KCl) were made with equal
377 percentages (3.6%) or equal molarity (1,23 mOsmol/L) to NaCl as shown in Table 2. Each solution was
378 mixed with Ad-GFP (MOI=250) and applied apically to airway epithelia for 2 hours. GFP expression was
379 quantified by flow cytometry 5 days post-transduction.

380

381 *CXADR KO*

382 CRISPR/Cas9 was used to knockout the coxsackie and adenovirus receptor CXADR by nucleofection
383 (Lonza, Basel Switzerland). Well-differentiated human airway epithelia were lifted in TrypLE and
384 electroporated with ribonucleoproteins (RNP). CXADR Exon 4 was targeted with the following guide
385 RNAs: gRNA 1: ACGTAACATCTCGCACCTGAAGG and gRNA 2:
386 AGTACCTGCTAACCATGAAGTGG [17]. Alt-R S.p. HiFi Cas9 nuclease (1081058, IDT, Coralville, IA) was
387 mixed with crRNA and trcrRNA duplex to form the RNP. RNP and cells were mixed with the nucleofector
388 solution and electroporated using program U-024 and seeded in a 6 well plate in Pneumacult ExPlus media.
389 2 days later, cells were lifted and reseeded on a Corning 3413 collagen coated membranes. Cells were

maintained in Pneumacult ALI-M media for 3 weeks to differentiate. Ad5-GFP (CXADR-dependent) and Ad21-GFP (CXADR-independent) were formulated with DMEM or NaCl (3.6%) and applied to the apical surface of airway epithelia for 2 hours. GFP expression was quantified 5 days post-transduction by flow cytometry.

Inhibitors of endosomal acidification

Endosomal acidification inhibitors choloroquine (CQ) (200 μM) (C6628, Millipore Sigma, St. Louis, MO) and bafilomycin A1 (Baf A1) (1 μM) (19148, Millipore Sigma, St. Louis, MO) were used to pre-treat airway epithelia for 2 hours prior to Ad-GFP transduction in the presence or absence of NaCl (3.6%). GFP was quantified by flow cytometry 5 days post-transduction.

Pig explants transduction

0.25 cm^2 tracheal explants from newborn pigs were cultured on Surgifoam (1972, Ethicon, Raritan, NJ) for 3-5 days prior to transduction. Ad5-mcherry ($1x10^9$ TU), AAVH22-GFP ($2x10^{10}$ vg) were applied with or without NaCl (5%) to the apical surface of the trachea by inverting the trachea onto a tissue culture dish containing the viral solution for 2 hours then returned to an upright position on Surgifoam. 5 days post-transduction, tracheal explants were mounted on a microscope slide and imaged for fluorescent expression using confocal microscopy. Expression was quantified using Image J (FIJI) by measuring fluorescence intensity per high power field.

Adenoviral transduction of mouse airways

6-8 week old Balb/c mice were sedated with ketamine/xylazine (87.5 + 2.5 mg/kg). Ad-CMV-firefly luciferase was delivered intratracheally with $1x10^9$ TU formulated with NaCl (0.9% or 7% final). 5 days later mice were given intraperitoneal D-luciferin (50227, Millipore Sigma, St. Louis, MO) imaged for luminescence expression using the Xenogen IVIS-200.

Viral gene transfer in pig airways

Newborn pigs were sedated using isoflurane and viral vector formulated with NaCl (0.9% or 5% final) and aerosolized intratracheally using a MADgic Laryngo-Tracheal Mucosal Atomization Device (Teleflex, Morrisville, NC). Doses for each vector for each pig were as follows: $2.8x10^{10}$ infectious genomic units (IGU) of helper-dependent Ad-CMV-GFP and $7x10^8$ TU of GP64 HIV-CMV-GFP. 1 week later, pigs were humanely euthanized and lungs were analyzed for GFP expression. Lungs were systematically divided and fixed in 4% paraformaldehyde, subjected to a sucrose gradient, and embedded in OCT for cryosectioning.

Sectioned lung slides were mounted using DAPI and imaged using a Keyence All-in-one Fluorescence Microscope BZ-X series (Osaka, Japan).

Statistics

Student's two-tailed t-test, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, or two-way ANOVA with Dunnett's multiple comparison test were used to analyze differences in mean values between groups. Results are expressed as mean ± SEM. P values ≤ 0.05 were considered significant. R^2 values in Fig. 1E represent exponential growth of nonlinear regression.

Tonicity	*Isotonic*	*Hypertonic*						
	DMEM							
% NaCl	0.64%	1%	2%	3%	4%	5%	6%	7%
Molarity	110 mM	170 mM	340 mM	510 mM	680 mM	860 mM	1.03 mM	1.2 M
Osmolarity	219 mOsmol/L	342 mOsmol/L	684 mOsmol/L	1,027 mOsmol/L	1,369 mOsmol/L	1,711 mOsmol/L	2,053 mOsmol/L	2,693 mOsmol/L
% KCl	0.4%	1%	2%	3%	4%	5%	6%	7%
Molarity	50 mM	130 mM	270 mM	400 mM	540 mM	670 mM	800 mM	940 mM
Osmolarity	107 mOsmol/L	268 mOsmol/L	537 mOsmol/L	805 mOsmol/L	1,073 mOsmol/L	1,341 mOsmol/L	1,610 mOsmol/L	1,878 mOsmol/L
% mannitol		1%	2%	3%	4%	5%	6%	7%
Molarity		55 mM	110 mM	170 mM	220 mM	270 mM	330 mM	380 mM
Osmolarity		55 mOsmol/L	110 mOsmol/L	170 mOsmol/L	220 mOsmol/L	270 mOsmol/L	330 mOsmol/L	380 mOsmol/L
% NMDG gluconate		1%	2%	3%	4%	5%	6%	7%

Molarity		50 mM	100 mM	150 mM	200 mM	260 mM	310 mM	360 mM
Osmolarity		342 mOsmol/L	684 mOsmol/L	1,027 mOsmol/L	1,369 mOsmol/L	1,711 mOsmol/L	2,053 mOsmol/L	3,693 mOsmol/L

Table 1: Molarity and osmolarity of NaCl, KCl, mannitol, and NMDG up to 7%. Tonicity, molarity, and osmolarity of DMEM and 1-7% NaCl, KCl, mannitol, and N-methyl-D-glucamine gluconate (NMDG). Hypertonic indicated a NaCl solution >0.9% DMEM has additional inorganic salts (i.e., $CaCl_2$, $MgSO_4$, KCl, and $NaHCO_3$) and is considered isotonic.

	3.6% NaCl	**0.9% NaCl**	**0.9% NaCl +mannitol**	**3.6% NaGluconate**	**3.6% NaCl**
Molecular weight	58.44 g/mol	58.44 g/mol	Mannitol: 182.172 g/mol	590 g/mol	74.55 g/ml
Tonicity (%)	3.6%	0.9%	11.2%	3.6%	3.6%
Molarity	620 mM	150 mM	620 mM	61 mM	480 mM
Osmolarity	1,230 mOsmol/L	310 mOsmol/L	1,230 mOsmol/L	120 mOsmol/L	970 mOsmol/L

Table 2. Molarity and osmolarity of ionic and non-ionic osmolytes. Molecular weight, tonicity, molarity and osmolarity for NaCl (0.9% and 3.6%), mannitol, NaGluconate, and KCl are presented.

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Investigation: ALC
Visualization: ALC, PBM, PLS
Funding acquisition: PBM, PLS
Project administration: ALC
Supervision: PBM, PLS
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Writing – review & editing: ALC, PBM, PLS

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Figures

Fig. 1. **Increasing saline tonicity enhances apical Ad gene transfer in airway epithelia.** (**A**) Ad-CMV-eGFP (MOI=250) was co-delivered to primary human airway epithelial cultures with DMEM or NaCl ranging from 1-7% (final concentration) and imaged 5 days post-transduction. 0.33 cm^2 transwells were imaged at 2X. (**B**) Percentage of GFP+ cells in each condition quantified by flow cytometry. (**C**) Percentages of GFP+ cells by cell type from 1-7% NaCl. (**D**) Tight junction permeability was measured by the passage of dextran from the apical to basolateral media. Airway epithelial cells were left untreated or treated with NaCl (1-7%) for 2 hours. Following the treatment, apical dextran was applied and basolateral media was collected 30 minutes later and measured for fluorescent units were measured using a plate reader. (**E**) Correlative %GFP (R^2=0.75). from Fig. 1B and Dextran Permeability (R^2=0.86) from Fig. 1D were plotted together. N=3, *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005. Statistical differences were determined by one-way ANOVA.

Fig. 2. Apically applied Ad-CFTR + 3.6% NaCl restores anion transport defect in CF airway epithelia. (**A, B**) Short circuit current measurements of CF airway epithelia alone or treated with Ad-CFTR or Ad-CFTR + 3.6% NaCl (MOI=250) compared to non-CF. Responses to Forskolin and IBMX (F&I) and GlyH-101 (GlyH) are reported. (**C, D**) Short circuit conductance in response to F&I and GlyH N=5. (**E**) Representative images of parallel CF cultures transduced with Ad-GFP (MOI=250) and (**F**) quantified by flow cytometry (N=3). Inset: representative western blot of CF HAE untreated or transduced with Ad-CFTR or Ad-CFTR + 3.6% NaCl. Western blot was probed for CFTR (green) and Vinculin (red) (loading control). *p<0.05, **p<0.005, ***p<0.0005. Statistical differences were determined by Student's t-test or one-way ANOVA.

Fig. 3. Entry is dependent on ionic strength but not osmolarity, osmolytes, or pH. (**A**) Isotonic NaCl (0.9%), Isotonic NaCl + the non-ionic osmolyte mannitol with equivalent osmolarity (620 mM, 1,230 mOsmol/L) to 3.6% NaCl, NaGluconate (3.6%), and KCl (3.6%) were co-delivered apically to HAE with Ad-GFP (MOI=250) for 2 hours. (**B**) 5 days post-transduction, cells were imaged and GFP expression was quantified by flow cytometry. (**C**) Ad-GFP was co-delivered with NaCl (3.6%) at pH 5, 6, 7, 7.4 and 8 for 2 hours. 5 day post-transduction GFP was quantified by flow cytometry. (**D**) KCl, mannitol, or N-methyl-D-glucamine (NMDG) gluconate 1-7% was formulated with Ad-GFP (MOI=250) and applied to the apical surface of HAE for 2 hours. Cultures were imaged 5 days post-transduction and GFP expression was quantified by flow cytometry for (**E**) KCl, (**F**) mannitol, or (**G**) NMDG gluconate. N=3, *p<0.05, ****p<0.00005. Statistical differences were determined by one-way ANOVA.

Fig. 4. NaCl-mediated transduction requires receptor and low pH entry step. Ad5 utilizes CXADR for entry and Ad21 utilizes CD46. (**A**) CRISPR/Cas9 with gRNA targeted to CXADR was electroporated into HAE and allowed to re-differentiate at air liquid interface for 3 weeks. In parallel with non-electroporated cells from the same donor, Ad5-GFP and Ad21-GFP were applied apically to HAE with or without NaCl for 2 hours. Cultures were imaged 5 days post-transduction and representative images are shown. (**B**) GFP was quantified by flow cytometry. Inset: Western blot for CXADR in parallel on CXADR KO or scrambled airway epithelial cultures. (**C**) HAE were pre-treated with endosomal acidification inhibitors chloroquine (CQ) (200 μM) or bafilomycin A1 (1 μM) for 2 hours. Next, Ad5-GFP (MOI=250) was delivered with or without NaCl (3.6%) for 2 hours. GFP expression was quantified by flow 5 days post-transduction. N=3, *p<0.05, **p<0.005, ****p<0.00005. Statistical differences were determined by one-way ANOVA.

Fig. 5. Increasing saline tonicity enhances GP64 pseudotyped lentiviral and AAV transduction. (**A**) GP64 pseudotyped HIV-CMV-eGFP (MOI=50) was co-delivered to airway epithelia with DMEM,

3.5%, 4%, or 4.5% NaCl for 2 hours and then removed. 1 week post-transduction GFP expression was quantified by flow cytometry. (**B**) Parallel CF cultures were transduced with GP64 HIV-CMV-eGFP. GFP expression was visualized by fluorescence microscopy and quantified by flow cytometry 5 days later. (**C, D**) As described in Figure 2, CF airway epithelia were transduced with GP64-HIV-CFTR (MOI=50) formulated with DMEM or 4.5% NaCl for 2 hours and incubated for 1 week. Short circuit current of F&I and GlyH in untreated or treated CF cells compared to non-CF. (**E**) AAV with the 2.5T capsid expressing CMV-eGFP was co-delivered (MOI=100,000) with DMEM or 4.5% NaCl for 2 hours. Airway cells were pre-treated with doxorubicin (1 μM) for 24 hours prior to transduction. 2 weeks post-transduction, airway cultures were imaged and GFP was quantified by flow cytometry. (**F, G**) CF airway cultures were treated overnight with doxorubicin (1 μM) and the next day cells were treated with AAV2.5T-CFTR alone or with 4.5% NaCl for 2 hours. Two weeks post-transduction, electrical properties were measured by Ussing chambers as described in (C, D). N=3, *p<0.05, **p<0.005. Statistical differences were determined by Student's t-test or one-way ANOVA.

Fig. 6. Increased saline tonicity enhances transduction of mouse and pig airways. (**A**) 6-8 week old Balb/c mice received intratracheal Ad-luciferase formulated with NaCl (0.9% or 7% final concentration). Mice were imaged by IVIS 5 days after delivery. N=5. (**B-G**) Ad-GFP was formulated with PBS or 10% NaCl (5% final concentration) and aerosolized intratracheally to newborn pigs. 5 days after delivery lungs were divided into 20 regions (**B**) as previously reported [2]. (**C**) Representative fluorescent dissecting scope images of GFP expression. (**D**) Individual lung regions were scored using a dissecting fluorescent microscope (0= no expression, 1= low expression, 2=moderate expression, and 3= high expression). (**E**) Combined GFP+ scores. (**F**) Lungs were fixed, processed, sectioned, and counterstained with DAPI. Representative 2X and 10X images from 5% NaCl condition shown. (**G-I**) GP64 HIV-GFP formulated with PBS or NaCl (5% final concentration) was aerosolized to newborn pigs intratracheally. One week later, lungs were collected as described in B. Lobe regions were scored for GFP expression and representative images of fluorescent dissecting microscope shown. Pig experiments: n=1 animal per condition. 10 slides per lung were evaluated. **p<0.005. ****p<0.00005. Statistical differences were determined by Student's t-test.

Supplemental Fig. 1. Clinically approved agents decrease transepithelial resistance but only hypertonic saline (3.6% NaCl) enhances gene transfer. (**A**) Transepithelial electrical resistance (TEER) of HAE was measured using an ohmmeter (normalized to 0.33 cm^2 transwell area) after 5 minute incubation with indicated reagents: Ultraser G (USG), Dulbecco's Modified Eagle Medium (DMEM), phosphate buffered saline (PBS) with (+/+) or without (-/-) calcium and magnesium, 0.1% lysophosphatidylcholine (LPC), 250 mM EGTA, 5 μM doxorubicin (Dox), surfactant (Infasurf) (35

mg/ml), or 3.6% NaCl. (**B**) Ad-GFP (MOI=250) was applied apically to HAE with PBS -/-, LPC, EGTA, Dox, Surfactant, or 3.6% NaCl for 2 hours. Cultures were imaged 5 days post-transduction. ****p<0.00005.

Supplemental Fig. 2. Effects of NaCl treatment on primary human airway epithelia. (**A**) Distribution of cell types with increasing %NaCl 5 days after treatment. Nerve growth factor receptor (NGFR)=basal cells, CD66c=secretory cells, α-tubulin=ciliated cells. (**B**) HAE from (**A**) were treated with LIVE/DEAD followed by immediated fixation prior to assaying by flow cytometry. %Live cells reported for 1-7% NaCl. (**C**) Cytotoxicity as measured by %LDH release for 1-7% NaCl. (**D**) SEM images after 2 hour treatment with DMEM or NaCl (3.6%). N=3, p<0.05. Scale bar=50 μm. Arrows indicate cilia.

Supplemental Fig. 3. NaCl-mediated entry of Ad-GFP is time dependent. (**A**) Experiment schematic. Ad-GFP (green bar) was applied for 2 hours with 3.6% NaCl (gray bar) present at 120, 60, 30, 15, or 5 minutes. Parallel cultures were incubated with 3.6% NaCl for 2 hours with Ad-GFP present for 120, 60, 30, 15, or 5 minutes. The 2 hour co-treatment group was shared between groups. (**B, C**) Images were acquired 5 days post-transduction. Representative fluorescence microscopy images at each time interval. (**D**) GFP quantification by flow cytometry. N=3, *p<0.05, ***p<0.0005. Statistical differences were determined by one-way ANOVA.

Supplemental Fig. 4. NaCl-mediated entry does not enhance all lentiviral pseudotypes. Baculovirus glycoprotein 64 (GP64), Vesicular Stomatitis Virus (VSVG), Jaagsiekte Sheep Retrovirus (JSRV), Baboon Endogenous Virus (BaEV) pseudotyped HIV-GFP (MOI=10) were treated for 2 hours without and with 4.5% NaCl. GFP expression was quantified 5 days later by flow cytometry. N=3, *p<0.05, **p<0.005.

Supplemental Fig. 5. NaCl formulated with Ad and AAV enhances transduction in pig tracheal explants. Each vector (Ad-mCherry $1x10^9$ TU; AAVH22-GFP $2x10^{10}$ vg) was formulated with NaCl (5% final concentration) and applied to the apical surface of pig tracheal explants for 2 hours. (**A**) mCherry or GFP fluorescence expression was visualized 5 days post-transduction. (**B**) Images were quantified using ImageJ and reported as fluorescent intensity per high power field (HPF). N=5, *p<0.05, **p<0.005.

Fig. 1

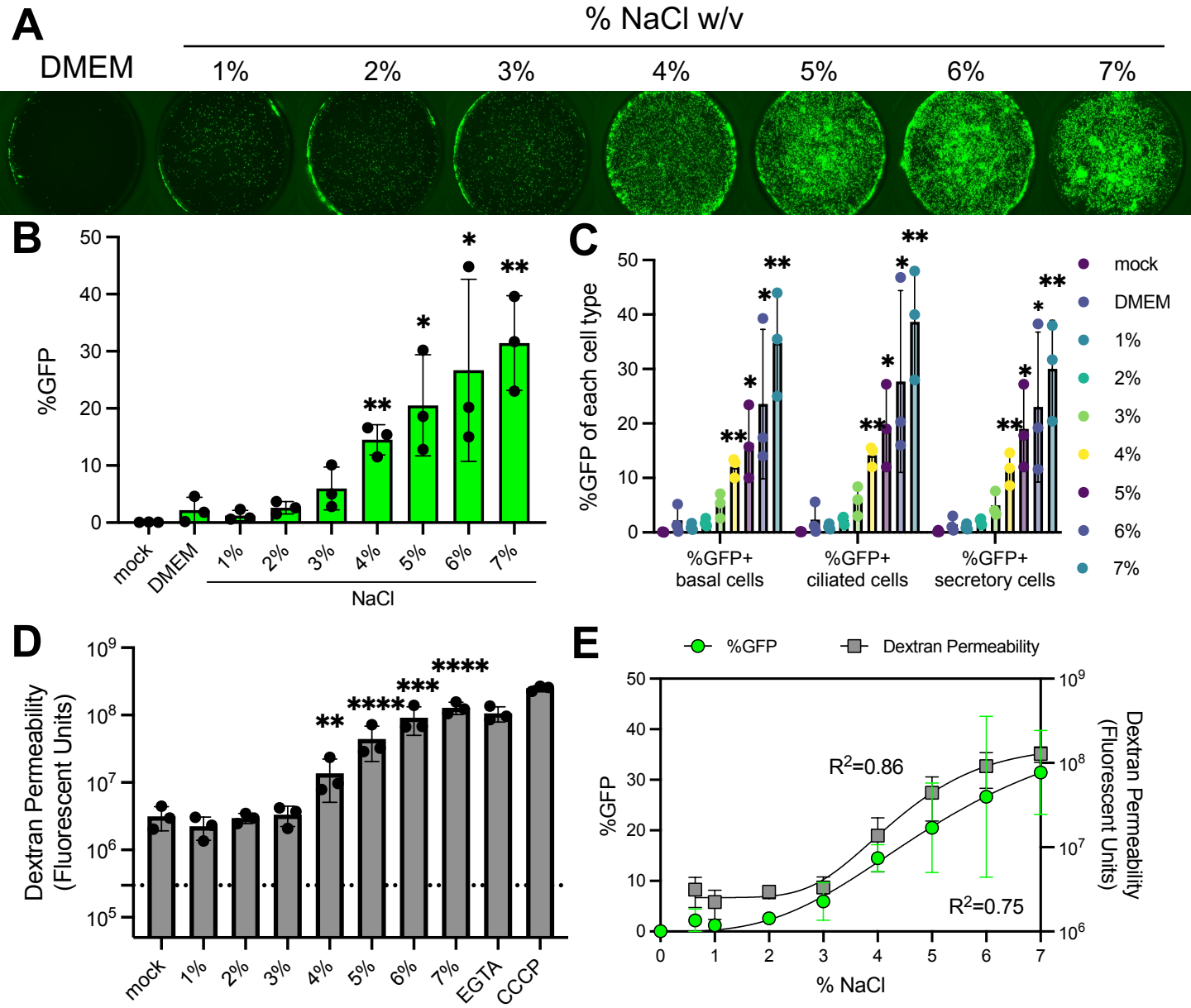

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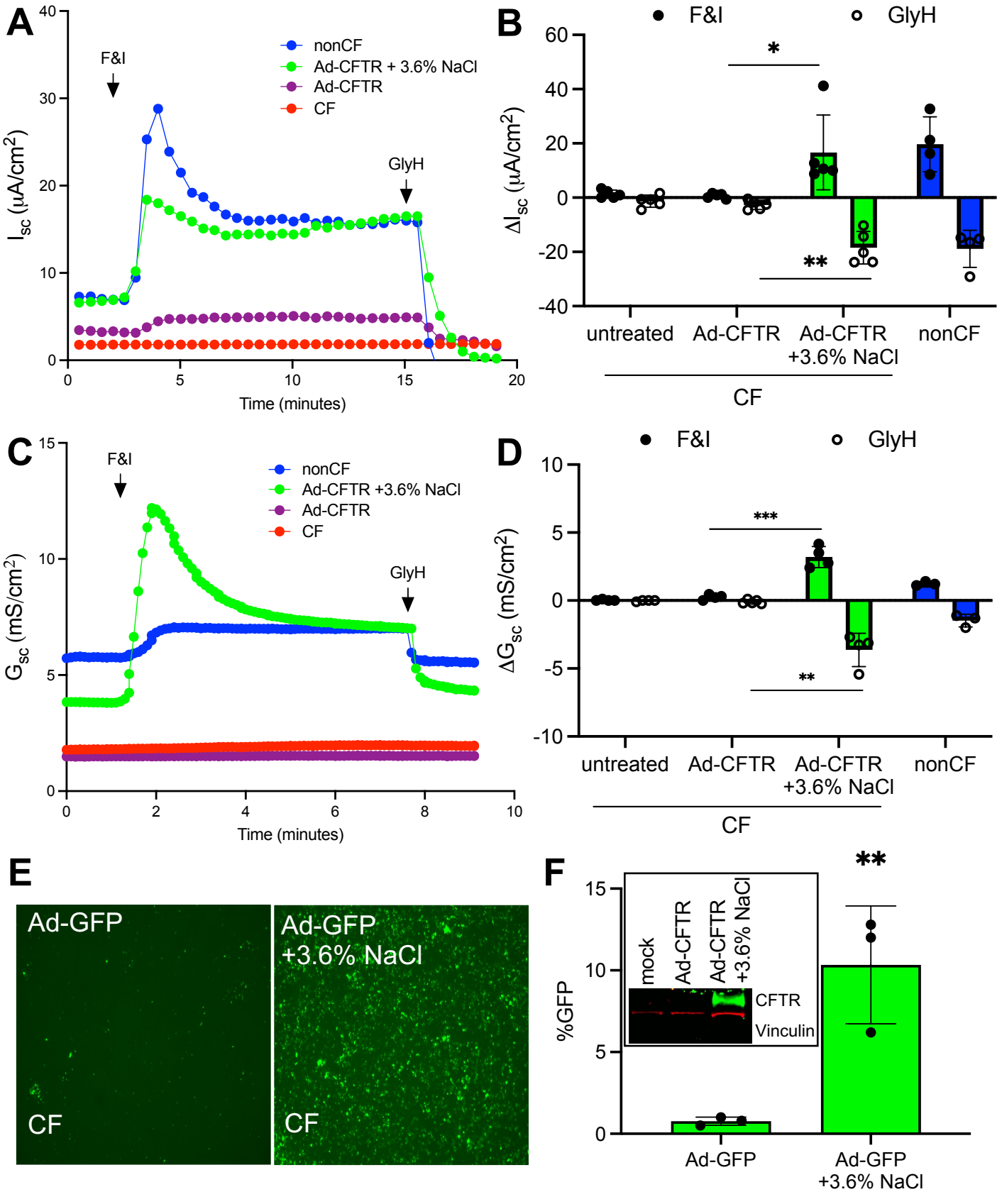
Fig. 2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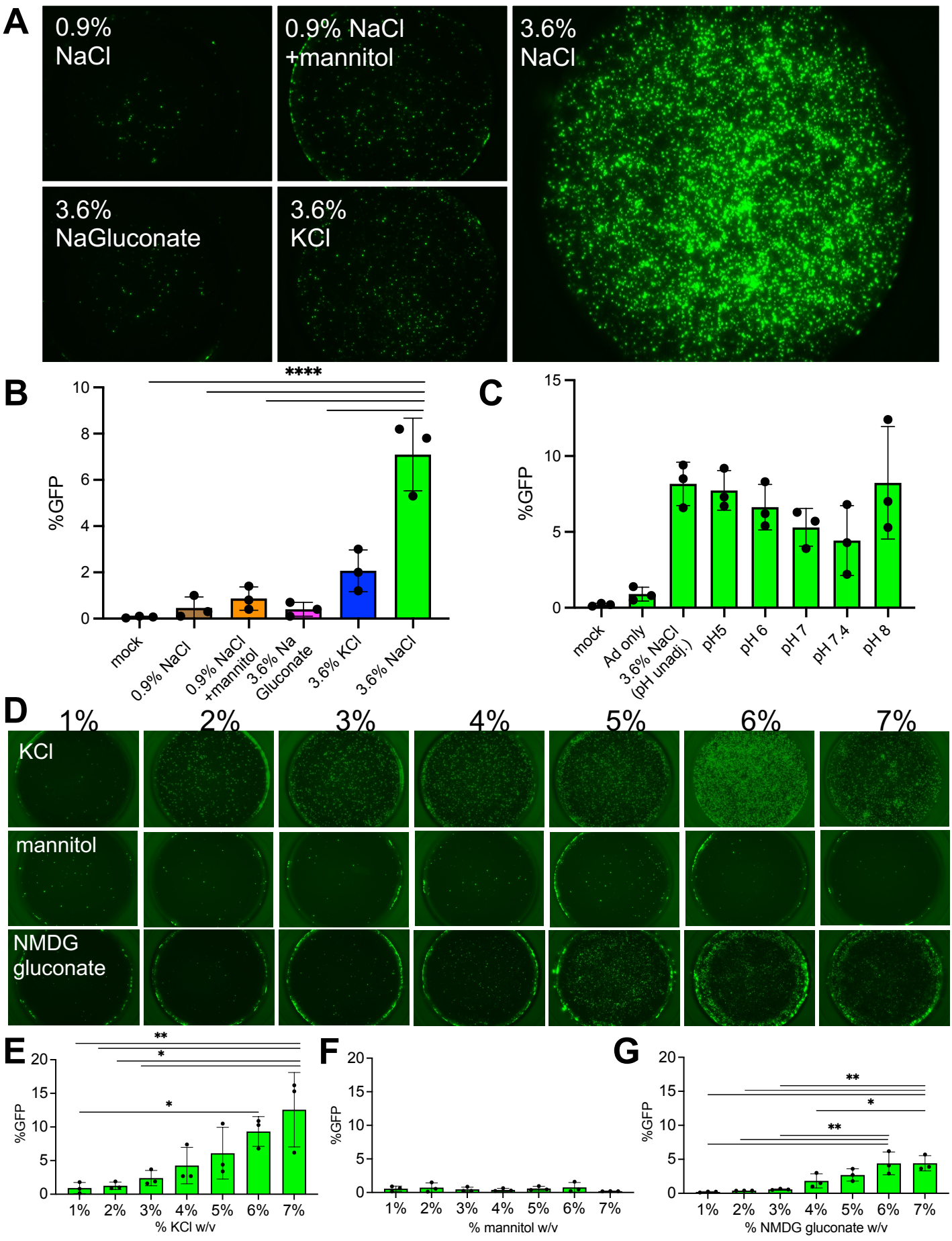
CFTR
Vinculin
Ad-GFP
Ad-GFP +3.6% NaCl

Fig. 2. Apically applied Ad-CFTR + 3.6% NaCl restores anion transport defect in CF airway epithelia. (**A, B**) Short circuit current measurements of CF airway epithelia alone or treated with Ad-CFTR or Ad-CFTR + 3.6% NaCl (MOI=250) compared to non-CF. Responses to Forskolin and IBMX (F&I) and GlyH-101 (GlyH) are reported. (**C, D**) Short circuit conductance in response to F&I and GlyH N=5. (**E**) Representative images of parallel CF cultures transduced with Ad-GFP (MOI=250) and (**F**) quantified by flow cytometry (N=3). Inset: representative western blot of CF HAE untreated or transduced with Ad-CFTR or Ad-CFTR + 3.6% NaCl. Western blot was probed for CFTR (green) and Vinculin (red) (loading control). *$p<0.05$, **$p<0.005$, ***$p<0.0005$. Statistical differences were determined by Student's t-test or one-way ANOVA.

Fig. 3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0.9% NaCl
0.9% NaCl +mannitol
3.6% NaCl
3.6% NaGluconate
3.6% KCl
B
****
%GFP
mock
0.9% NaCl
0.9% NaCl +mannitol
3.6% Na Gluconate
3.6% KCl
3.6% NaCl
C
%GFP
mock
Ad only
3.6% NaCl (pH unadj.)
pH5
pH 6
pH 7
pH 7.4
pH 8
D
1%
2%
3%
4%
5%
6%
7%
KCl
mannitol
NMDG gluconate
E
%GFP
% KCl w/v
F
%GFP
% mannitol w/v
G
%GFP
% NMDG gluconate w/v

Fig. 3. Entry is dependent on ionic strength but not osmolarity, osmolytes, or pH. (**A**) Isotonic NaCl (0.9%), Isotonic NaCl + the non-ionic osmolyte mannitol with equivalent osmolarity (0.62 M) to 3.6% NaCl, NaGluconate (3.6%), and KCl (3.6%) were co-delivered apically to HAE with Ad-GFP (MOI=250) for 2 hours. 5 days post-transduction, cells were imaged (**B**) and GFP expression was quantified by flow cytometry. (**C**) Ad-GFP was co-delivered with NaCl (3.6%) at pH 5, 6, 7, 7.4 and 8 for 2 hours. 5 day post-transduction GFP was quantified by flow cytometry. (**D**) KCl, mannitol, or N-methyl-D-glucamine gluconate (NMDG) 1-7% was formulated with Ad-GFP (MOI=250) and applied to the apical surface of HAE for 2 hours. Cultures were imaged 5 days post-transduction and GFP expression was quantified by flow cytometry for (**E**) KCl, (**F**) mannitol, or (**G**) NMDG. N=3, *$p<0.05$, ****$p<0.00005$. Statistical differences were determined by one-way ANOVA.

Fig. 4

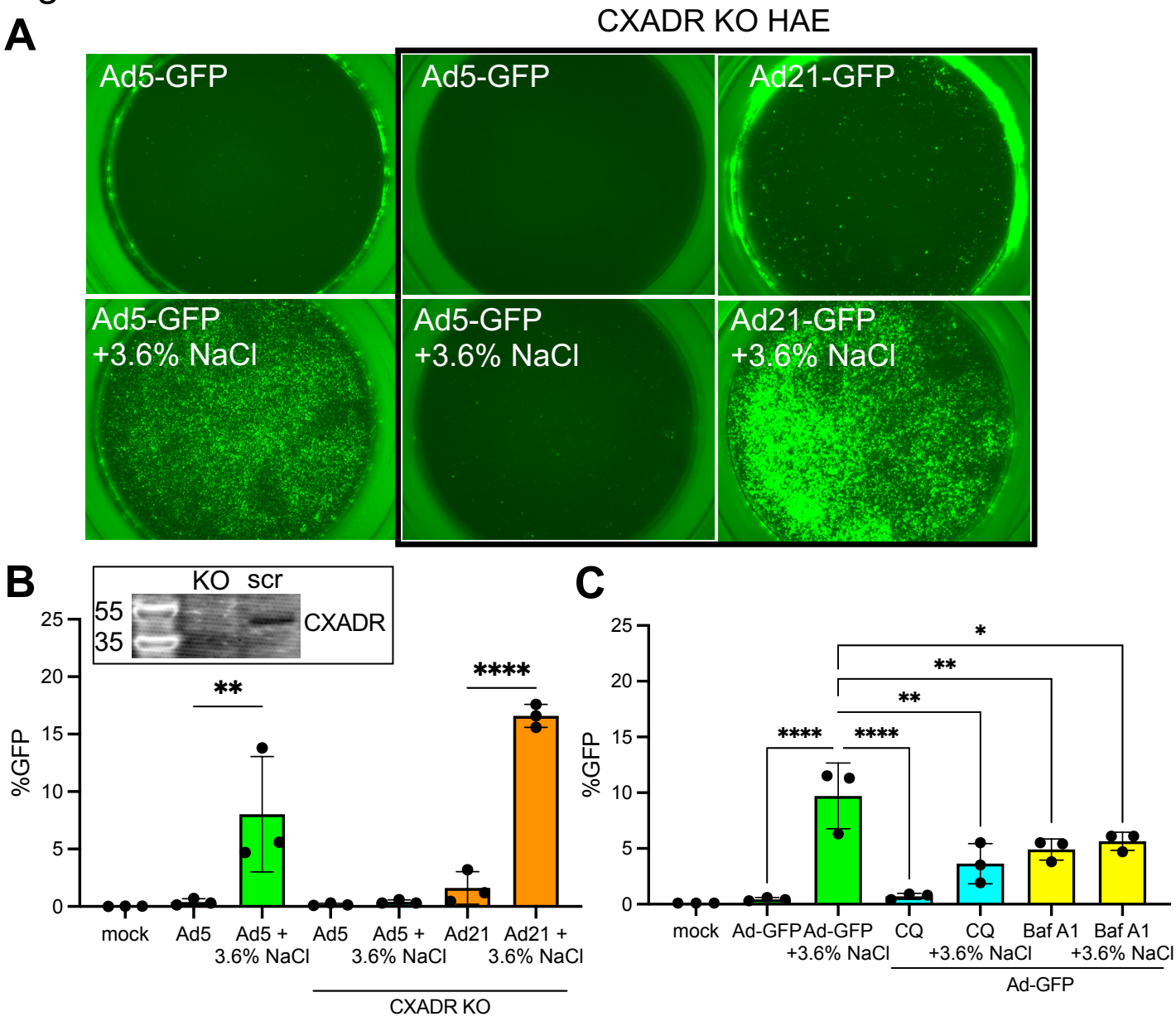


Fig. 4. NaCl-mediated transduction requires receptor and low pH entry step. Ad5 utilizes CXADR for entry and Ad21 utilizes CD46. (**A**) CRISPR/Cas9 with gRNA targeted to CXADR was electroporated into HAE and allowed to re-differentiate at air liquid interface for 3 weeks. In parallel with non-electroporated cells from the same donor, Ad5-GFP and Ad21-GFP were applied apically to HAE with or without NaCl for 2 hours. Cultures were imaged 5 days post-transduction and representative images are shown. (**B**) GFP was quantified by flow cytometry. Inset: Western blot for CXADR in parallel on CXADR KO or scrambled airway epithelial cultures. (**C**) HAE were pre-treated with endosomal acidification inhibitors chloroquine (CQ) (200 μM) or bafilomycin A1 (1 μM) for 2 hours. Next, Ad5-GFP (MOI=250) was delivered with or without NaCl (3.6%) for 2 hours. GFP expression was quantified by flow 5 days post-transduction. N=3, *p<0.05, **p<0.005, ****p<0.00005. Statistical differences were determined by one-way ANOVA.

Fig. 5

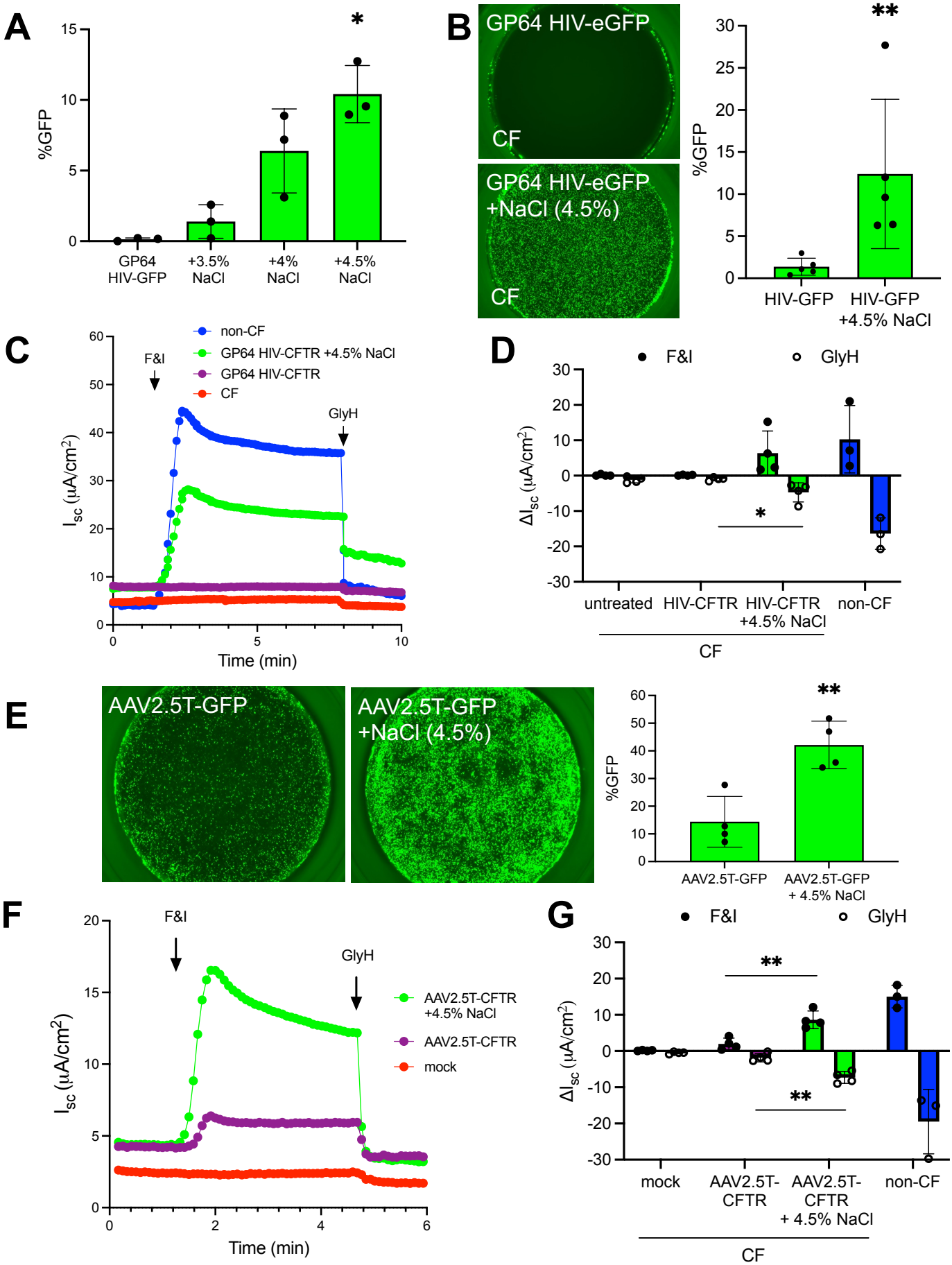

A
%GFP
GP64 HIV-GFP
+3.5% NaCl
+4% NaCl
+4.5% NaCl
B
GP64 HIV-eGFP
CF
GP64 HIV-eGFP +NaCl (4.5%)
CF
%GFP
HIV-GFP
HIV-GFP +4.5% NaCl
C
non-CF
GP64 HIV-CFTR +4.5% NaCl
GP64 HIV-CFTR
CF
F&I
GlyH
Isc (μA/cm2)
Time (min)
D
F&I
GlyH
ΔIsc (μA/cm2)
untreated
HIV-CFTR
HIV-CFTR +4.5% NaCl
non-CF
CF
E
AAV2.5T-GFP
AAV2.5T-GFP +NaCl (4.5%)
%GFP
AAV2.5T-GFP
AAV2.5T-GFP + 4.5% NaCl
F
F&I
GlyH
AAV2.5T-CFTR +4.5% NaCl
AAV2.5T-CFTR
mock
Isc (μA/cm2)
Time (min)
G
F&I
GlyH
ΔIsc (μA/cm2)
mock
AAV2.5T-CFTR
AAV2.5T-CFTR + 4.5% NaCl
non-CF
CF

Fig. 5. Increasing saline tonicity enhances GP64 pseudotyped lentiviral and AAV transduction. (**A**) GP64 pseudotyped HIV-CMV-eGFP (MOI=50) was co-delivered to airway epithelia with DMEM, 3.5%, 4%, or 4.5% NaCl for 2 hours and then removed. 1 week post-transduction, cells were imaged using fluorescence microscopy and GFP expression was quantified by flow cytometry. (**B**) Parallel CF cultures were transduced with GP64 HIV-CMV-eGFP and quantified by flow cytometry. As described in Figure 2, CF airway epithelia were transduced with GP64-HIV-CFTR formulated with DMEM or 4.5% NaCl for 2 hours and incubated for 1 week. (**C, D**) Short circuit current of F&I and GlyH in untreated or treated CF cells compared to non-CF. (**E**) AAV with the 2.5T capsid expressing CMV-eGFP was co-delivered (MOI=100,000) with DMEM or 4.5% NaCl for 2 hours. Airway cells were pre-treated with doxorubicin (1 μM) for 24 hours prior to transduction. 2 weeks post-transduction, airway cultures were imaged and GFP was quantified by flow cytometry. (**F, G**) CF airway cultures were treated overnight with doxorubicin (1 μM) and the next day cells were treated with AAV2.5T-CFTR alone or with 4.5% NaCl for 2 hours. Two weeks post-transduction, electrical properties were measured by Ussing chambers as described in (C, D). N=3, *$p<0.05$, **$p<0.005$. Statistical differences were determined by Student's t-test or one-way ANOVA.

Fig. 6

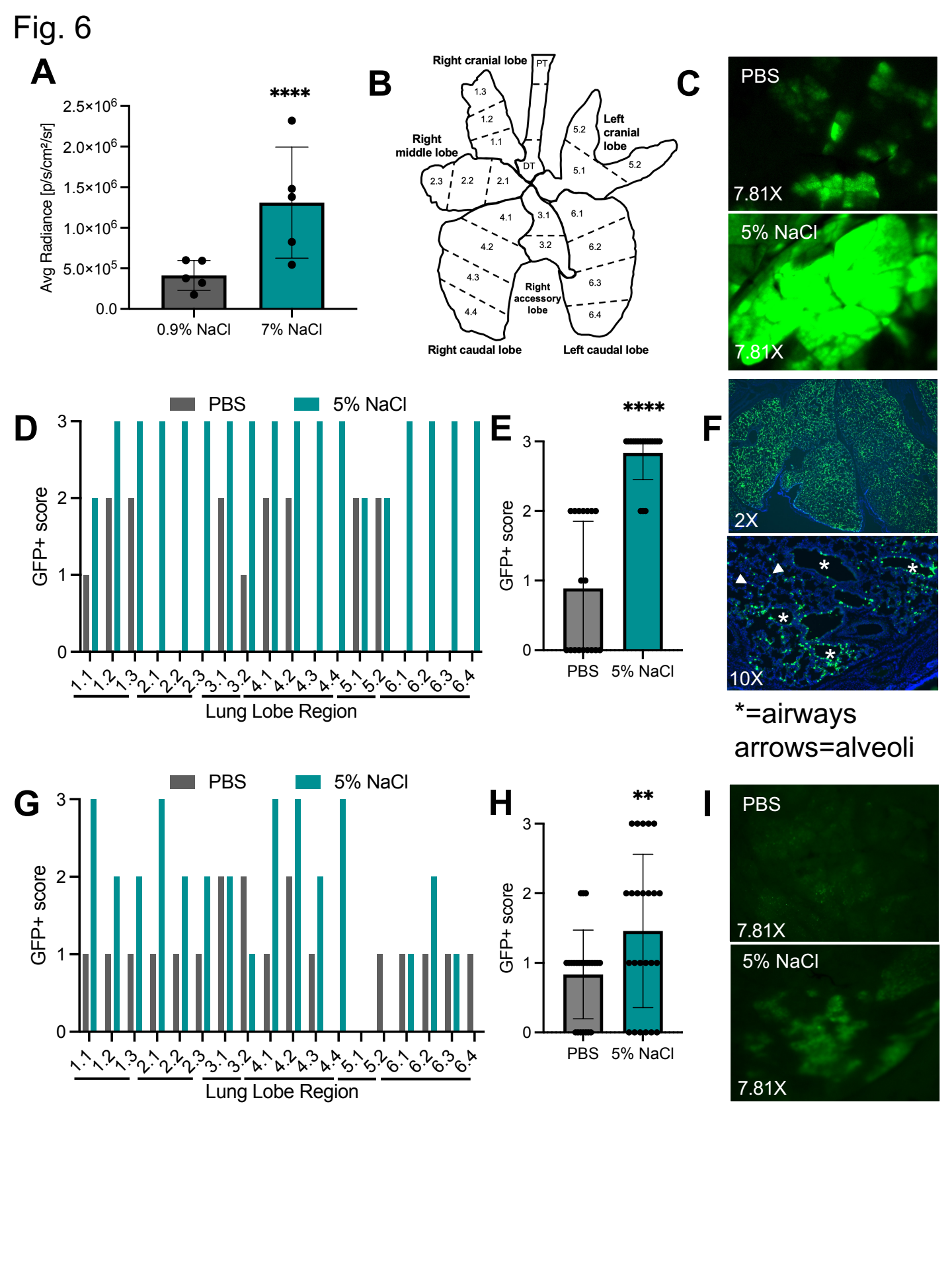
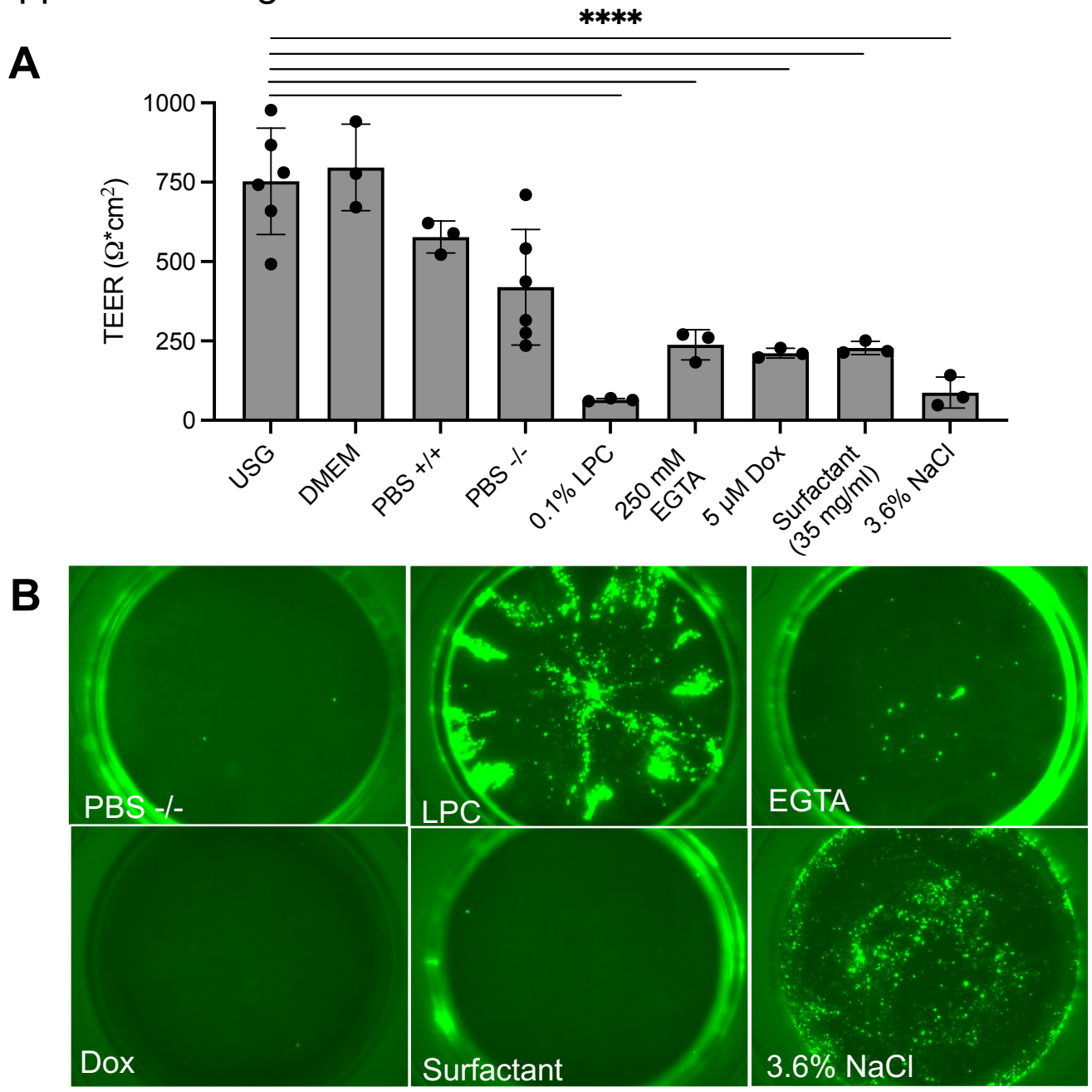

A
****
Avg Radiance [p/s/cm²/sr]
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
0.9% NaCl
7% NaCl
B
Right cranial lobe
PT
Left cranial lobe
Right middle lobe
DT
Right accessory lobe
Right caudal lobe
Left caudal lobe
C
PBS
7.81X
5% NaCl
7.81X
D
PBS
5% NaCl
GFP+ score
Lung Lobe Region
E
****
GFP+ score
PBS
5% NaCl
F
2X
10X
*=airways
arrows=alveoli
G
PBS
5% NaCl
GFP+ score
Lung Lobe Region
H
**
GFP+ score
PBS
5% NaCl
I
PBS
7.81X
5% NaCl
7.81X

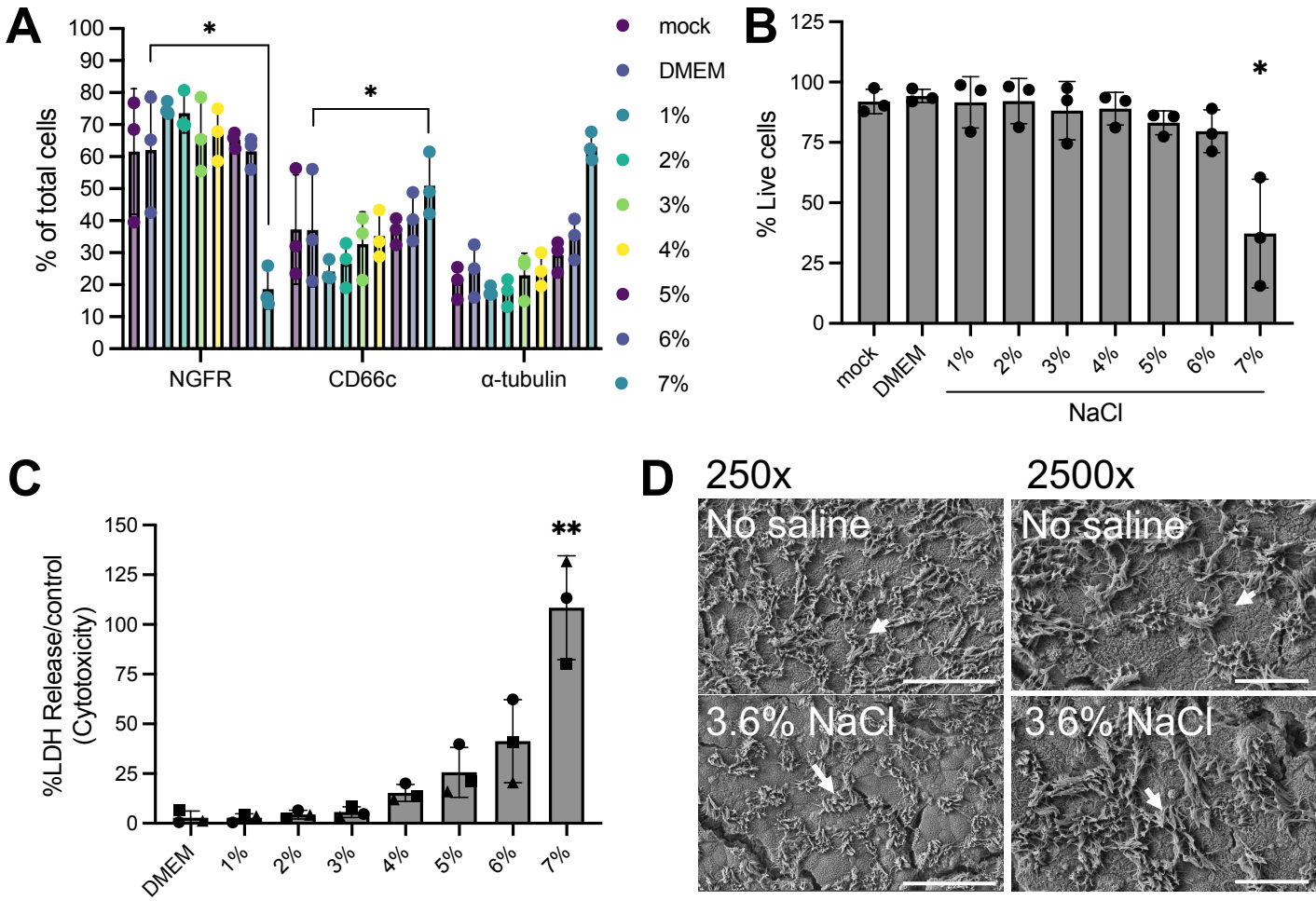
Fig. 6. Increased saline tonicity enhances transduction of mouse and pig airways. (**A**) 6-8 week old Balb/c mice received intratracheal Ad-luciferase formulated with NaCl (0.9% or 7% final concentration). Mice were imaged by IVIS 5 days after delivery. N=5. (**B-G**) Ad-GFP was formulated with PBS or 10% NaCl (5% final concentration) and aerosolized intratracheally to newborn pigs. 5 days after delivery lungs were divided into 20 regions (**B**) as previously reported [2]. (**C**) Representative fluorescent dissecting scope images of GFP expression. (**D**) Individual lung regions were scored using a dissecting fluorescent microscope (0= no expression, 1= low expression, 2=moderate expression, and 3= high expression). (**E**) Combined GFP+ scores. (**F**) Lungs were fixed, processed, sectioned, and counterstained with DAPI. Representative 2X and 10X images from 5% NaCl condition shown. (**G-I**) GP64 HIV-GFP formulated with PBS or NaCl (5% final concentration) was aerosolized to newborn pigs intratracheally. One week later, lungs were collected as described in B. Lobe regions were scored for GFP expression and representative images of fluorescent dissecting microscope shown. Pig experiments: n=1 animal per condition. 10 slides per lung were evaluated. **$p<0.005$. ****$p<0.00005$. Statistical differences were determined by Student's t-test.

Supplemental Fig. 1



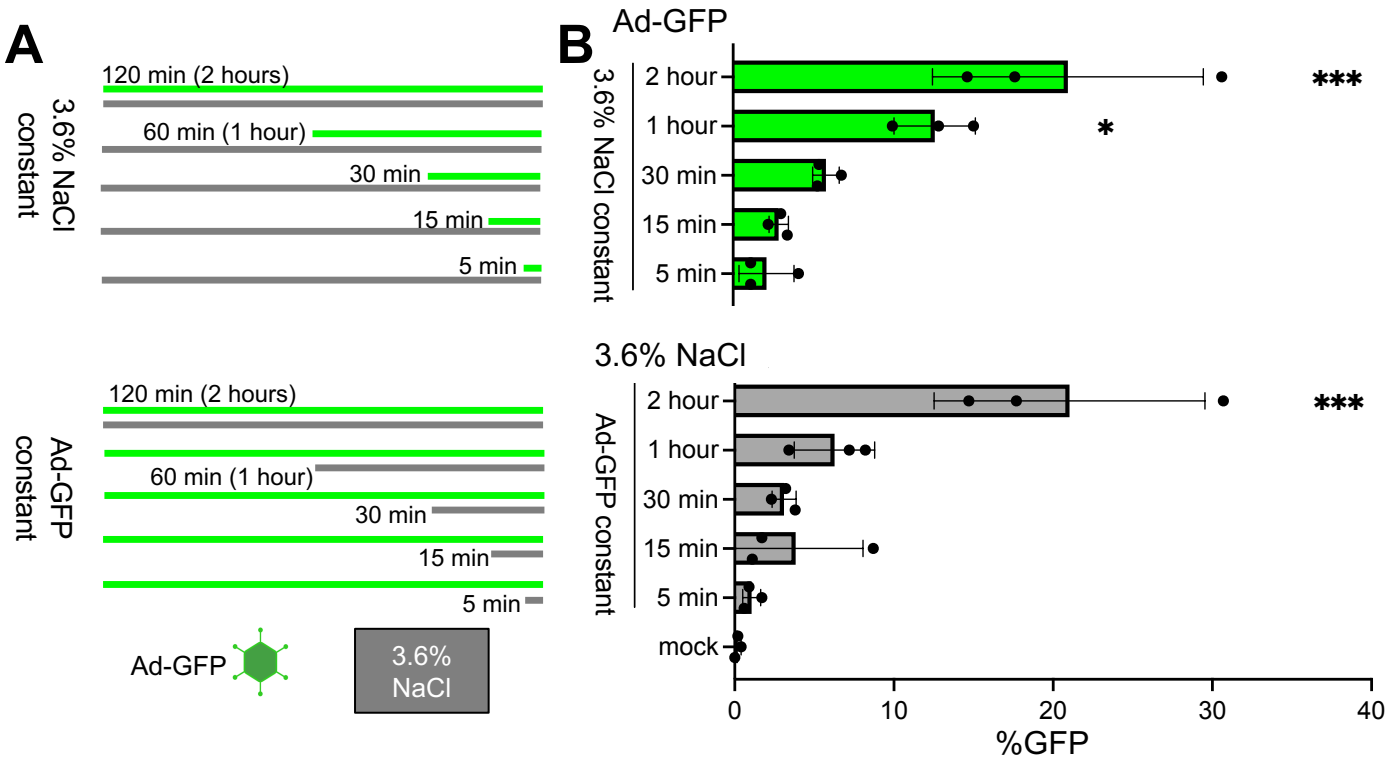
Supplemental Fig. 1. Clinically approved agents decrease transepithelial resistance but only hypertonic saline (3.6% NaCl) enhances gene transfer. (**A**) Transepithelial electrical resistance (TEER) of HAE was measured using an ohmmeter (normalized to 0.33 cm^2 transwell area) after 5 minute incubation with indicated reagents: Ultraser G (USG), Dulbecco's Modified Eagle Medium (DMEM), phosphate buffered saline (PBS) with (+/+) or without (-/-) calcium and magnesium, 0.1% LPC, 250 mM EGTA, 5 µM doxorubicin (Dox), surfactant (Infasurf)(35 mg/ml), or 3.6% NaCl. (**B**) Ad-GFP (MOI=250) was applied apically to HAE with PBS -/-, LPC, EGTA, Dox, Surfactant, or 3.6% NaCl for 2 hours. Cultures were imaged 5 days post-transduction. ****$p<0.00005$.

Supplemental Fig. 2



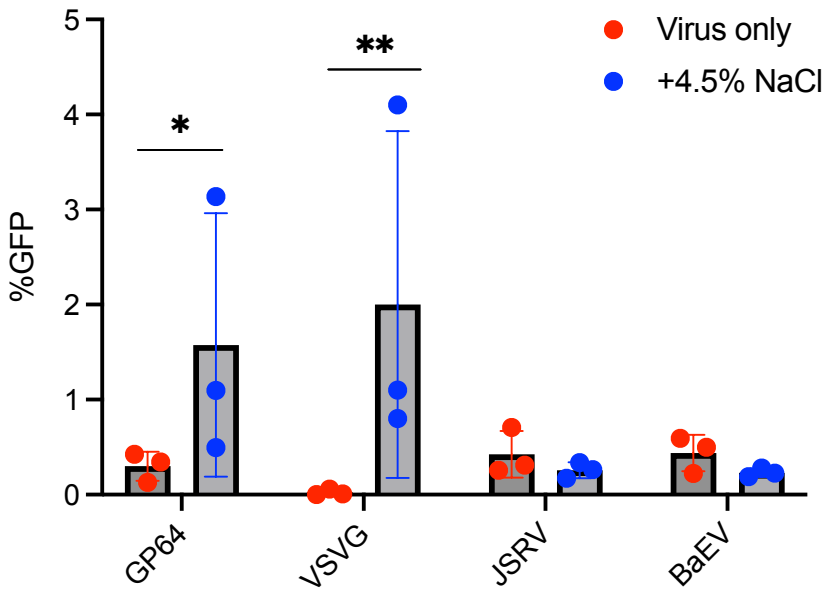
Supplemental Fig. 2. Effects of NaCl treatment on primary human airway epithelia. (**A**) Distribution of cell types with increasing %NaCl 5 days after treatment. Nerve growth factor receptor (NGFR)=basal cells, CD66c=secretory cells, α-tubulin=ciliated cells. (**B**) HAE from (**A**) were treated with LIVE/DEAD followed by immediated fixation prior to assaying by flow cytometry. %Live cells reported for 1-7% NaCl. (**C**) Cytotoxicity as measured by %LDH release for 1-7% NaCl. (**D**) SEM images after 2 hour treatment with DMEM or NaCl (3.6%). N=3, $p<0.05$. Scale bar=50 µm. Arrows indicate cilia.

Supplemental Fig. 3



Supplemental Fig. 3. NaCl-mediated entry of Ad-GFP is time dependent. (**A**) Experiment schematic. Ad-GFP (green bar) was applied for 2 hours with 3.6% NaCl (gray bar) present at 120, 60, 30, 15, or 5 minutes. Parallel cultures were incubated with 3.6% NaCl for 2 hours with Ad-GFP present for 120, 60, 30, 15, or 5 minutes. The 2 hour co-treatment group was shared between groups. (**B, C**) Images were acquired 5 days post-transduction. Representative fluorescence microscopy images at each time interval. (**D**) GFP quantification by flow cytometry. N=3, *p<0.05, ***p<0.0005. Statistical differences were determined by one-way ANOVA.

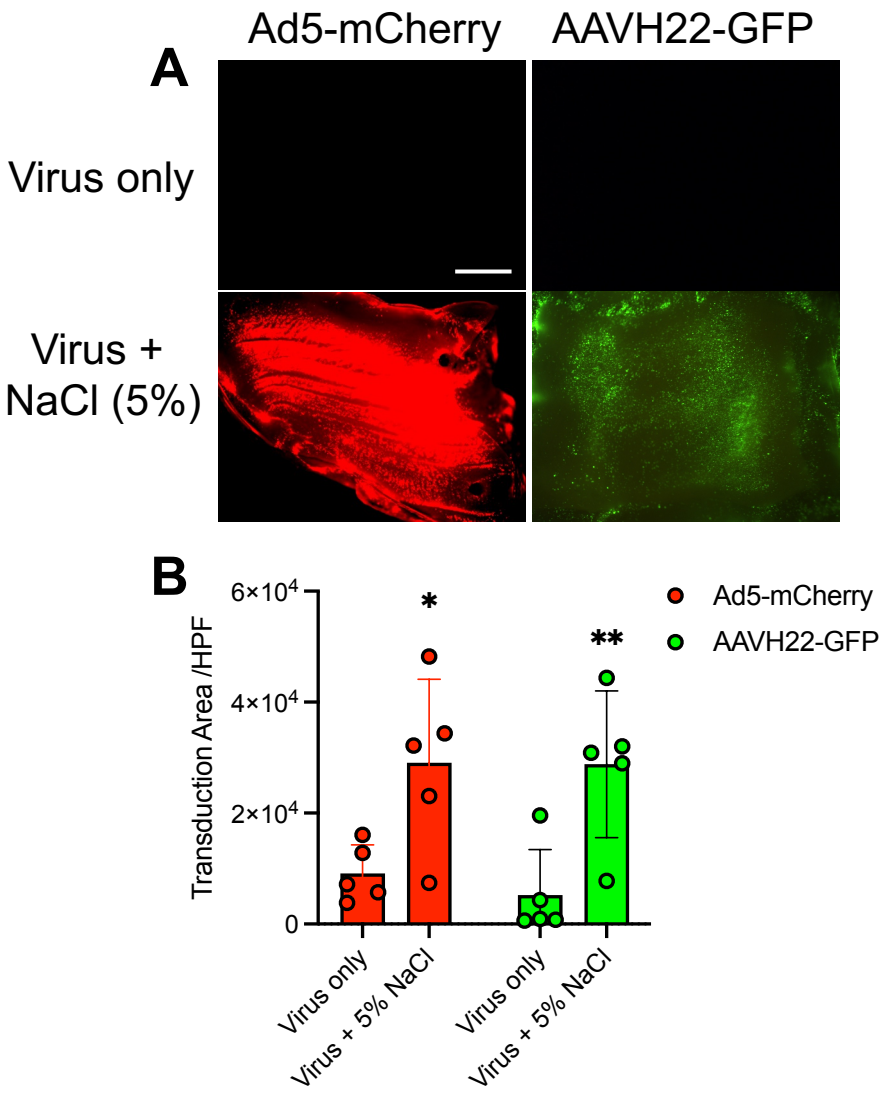
Supplemental Fig. 4



Supplemental Fig. 4. NaCl-mediated entry does not enhance all lentiviral pseudotypes.

Baculovirus glycoprotein 64 (GP64), Vesicular Stomatitis Virus (VSVG), Jaagsiekte Sheep Retrovirus (JSRV), Baboon Endogenous Virus (BaEV) pseudotyped HIV-GFP (MOI=10) were tested without and with 4.5% NaCl. N=3, *$p<0.05$, **$p<0.005$.

Supplemental Fig. 5



Supplemental Fig. 5. NaCl formulated with Ad and AAV enhances transduction in pig tracheal explants. Each vector (Ad-mCherry $1x10^9$ TU; AAVH22-GFP $2x10^{10}$ vg) was formulated with NaCl (5% final concentration) and applied to the apical surface of pig tracheal explants for 2 hours. (**A**) mCherry or GFP fluorescence expression was visualized 5 days post-transduction. (**B**) Images were quantified using ImageJ and reported as fluorescent intensity per high power field (HPF). N=5, *$p<0.05$, **$p<0.005$.