MATERIALS & METHODS

Polymer synthesis

PBAE polymers were synthesized as previously described.[1] Briefly, 1,4-butanediol diacrylate (Alfa Aesar, Haver Hill, MA) and 4-amino-1-butanol (Alfa Aesar) were reacted at a molar ratio of 1.1:1 at 95 °C for 20 hours to yield terminally acrylated polymers. Polymers were then precipitated and purified by cold anhydrous diethyl ether (Fisher Scientific, Pittsburgh, PA), dried under vacuum for at least 72 hours and characterized by nuclear magnetic resonance (NMR) to exhibit the molecular weight (MW) of 6 ± 0.2 kDa. The terminal acrylate groups were subsequently reacted with 30 molar equivalents of 2-(3-aminopropylamino)ethanol (Acros, Geel, Belgium) in tetrahydrofuran (THF) overnight at room temperature, followed by the cold ether precipitation/purification and vacuum-mediated solvent removal. The reaction was then confirmed by the absence of the diacrylate peaks in the NMR spectrum. The final PBAE polymers were dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml and stored at -20 °C until use.

For the synthesis of PEG-PBAE polymers, terminally acrylated polymers were synthesized and purified using the abovementioned steps but at a molar ratio of 1.2:1. We then estimated the MW to be 4 ± 0.2 kDa using NMR. Subsequently, the terminal acrylate groups were reacted with 30 molar equivalents of 1,3-diaminopropane in THF, and subsequently purified and dried as described above. These terminally aminated polymers were then reacted with 2.05 molar equivalents of methoxy-PEG-succinimidyl succinate (JenKem, Beijing, China) in THF overnight at room temperature, followed by purification and solvent-removal steps, to yield the final product of PEG-PBAE polymers. The PEG-PBAE polymers were dissolved in DMSO and stored at -20 °C until use.

Formulation and characterization of DNA-CP and DNA-MPP

The luciferase-encoding plasmid driven by a human β-actin promoter was produced by Copernicus Therapeutics. The GFP-encoding plasmid driven by a cytomegalovirus promoter was purchased from Clontech Laboratories, and the plasmid encoding shRNA against α-subunit of ENaC (shENaC; *Scnn1a* location 1498, target sequence ggcgaattattctcagttcca), driven by H1 promoter and cloned into the psi-H1^{TM} plasmid backbone, was purchased from Genecopoeia (Rockville, MD). The plasmids were expanded in appropriate competent *E.coli* strains following transformation and were isolated using Endofree Plasmid Giga Kit (Qiagen, Hilden, Germany). Downregulation of α-subunit of ENaC by the plasmid encoding shENaC was validated *in vitro* in M-1 mouse kidney epithelial cell line (ATCC, Manassas, VA) using Lipofectamine 3000 (Figure S2). For the assessment of DNA-CP and DNA-MPP distribution in mouse lungs, Label IT® Tracker Intracellular Nucleic Acid Localization Kit (Mirus Bio, Madison, WI) was used to fluorescently label plasmids with Cy3 fluorophores.

DNA-CP and DNA-MPP were formulated as previously described.[1 2] The polymer solution was prepared with PBAE only or a mixture of PBAE and PEG-PBAE (at a wt/wt ratio of 2:3 based on PBAE mass) for DNA-CP or DNA-MPP, respectively. To engineer DNA-loaded nanoparticles, five volumes of nucleic acids, including labeled or unlabeled plasmid DNA at 0.1 mg/ml, were added dropwise to one volume of a polymer solution at a PBAE-to-nucleic acid wt/wt ratio of 60:1 while vortexing. DNA-loaded nanoparticles were then washed with five volumes of

ultrapure distilled water at 950 xg for 8 minutes each time and concentrated to 0.5 mg/ml using Amicon Ultra Centrifugal Filters (100,000 molecular-weight cutoff; Millipore). We then measured hydrodynamic diameters and polydispersity indices of particles in water by dynamic light scattering (DLS), and particle ζ-potentials were measured in 10 mM NaCl at pH 7.0 by laser Doppler anemometry, using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). We then assessed the particle colloidal stability in a physiologically relevant lung environment by incubating DNA-CP and DNA-MPP in mouse BAL fluid at 37 °C and monitoring particle hydrodynamic diameters by DLS over time up to 4 hours. The measurement was prematurely discontinued for DNA-CP due to instantaneous aggregation upon the incubation in the BAL fluid (Figure 1). To confirm the stable compaction of different plasmids in DNA-MPP, we conducted conventional gel electrophoretic retardation assay. In parallel, protection of plasmid payloads by DNA-MPP was assessed by incubating DNA-MPP with 2 U of DNase I (ThermoFisher, Waltham, MA) for 15 minutes at 37 °C. Subsequently, DNase was deactivated by 50 mM EDTA (at 65 °C) and plasmid payloads were de-compacted from DNA-MPP by heparin (at a 3:1 w/w ratio of heparin to DNA at room temperature for 10 minutes; Sigma Aldrich, St. Louis, MO), followed by conventional gel electrophoresis. Gels were imaged using a Chemi-Doc imaging system (Bio-RAD, Hercules, CA).

Surface plasmon resonance binding assay

Adhesion of DNA-CP or DNA-MPP to porcine gastric mucin was investigated using a high-throughput surface plasmon resonance-based Biacore 3000 instrument (GE Healthcare, Marlborough, MA) at 25 °C, as previously described. [3-5] Isolated porcine gastric mucin was diluted in a sodium acetate buffer (pH 4; GE Healthcare, Marlborough, MA) to a final concentration of 50 μg/ml and conjugated at a final ligand response unit of 1127 to one of the flow channels of a CM5 Biacore chip using carbodiimide crosslinker chemistry, as per manufacturer's protocol. Subsequently, another flow path was activated and blocked using ethanolamine to serve as a reference for each binding run. The running buffer, 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 0.05% surfactant P-20 with 50 μM EDTA (HBS-P) was degassed prior to use.

For binding experiments, the DNA-CP or DNA-MPP were diluted in the running buffer and the extent of binding was assessed at a flow rate of 20 μl/min with an injection time of 3 minutes, followed by a 2.5 minute wait for dissociation prior to chip regeneration with 10 mM glycine (pH 1.75). Binding data was analyzed using Biacore 3000 Evaluation software where data from the reference flow path was subtracted from the experimental flow path to yield the final binding sensorgrams, as previously described.[3-5]

Animal studies – *in vivo* particle distribution and reporter transgene expression

Mice were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee. The *Scnn1b*-Tg mouse colony was maintained on a mixed genetic background (C3H/HeN x C57BL/6N) and individual newborn mice were genotyped (TransnetYX, Inc., Cordova, TN) to distinguish *Scnn1b*-Tg mice and wild-type littermates. Four-week old mice were used for all *in vivo* studies.

To assess the distribution of inhaled particles and transgene expression in the mouse lungs, a 50 μl suspension of DNA-CP or DNA-MPP carrying Cy3-labeled plasmids and unlabeled GFP-encoding plasmids, respectively, was intratracheally administered using a microsprayer (Model

IA-1C, Penn-Century, Wyndmoor, PA) at a plasmid concentration of 0.5 mg/ml (n = 3 mice per group). Mice were euthanized 1 and 48 hour(s) after the administration to evaluate the distribution of particles and transgene expression, respectively. For the particle distribution study, lungs were perfused with PBS, fixed with 4% paraformaldehyde overnight at 4 °C, and sequentially incubated in 10%, 20%, and 30% sucrose gradient solutions for two days each at 4 °C, prior to embedding in the optimal cutting temperature (OCT) compound. The lung tissues were cryosectioned using a CM1950 cyrostat (Leica Biosystems, Wetzler, Germany), followed by DAPI staining (ProLong® Gold Antifade with DAPI, ThermoFisher) and imaging with an inverted epifluorescence microscope (Axio Observer D1, Carl Zeiss, Oberkochen, Germany) at 10x magnification. For transgene expression distribution study, lungs were perfused with PBS and harvested. Lung tissues were then carefully filled with 1:1 mixture of PBS and OCT and immediately embedded in cryomolds. Following the cryosectioning, slides of lung tissues were fixed in ice-cold 100% acetone, blocked with Block-Aid agent (ThermoFisher), and immunologically stained overnight at 4 °C using an anti-GFP antibody tagged with Alexa 488 fluorophore (Biolegend, San Diego, CA) diluted in 1% bovine serum albumin/0.3% Triton X-100/PBS solution. After washing with PBS three times, the slides were stained with DAPI prior to imaging with a confocal LSM 710 microscope (Carl Zeiss) at 10x or 20x magnification. Lung sections from untreated mice were identically processed and used to determine the background fluorescence by adjusting the microscope settings. Image-based quantification of the distribution of particles and transgene expression was performed in a blinded manner using a custom-written JAVA code.

To assess the overall level of *in vivo* reporter transgene expression, a 50 μl suspension of DNA-CP or DNA-MPP carrying luciferase-encoding plasmids was intratracheally administered to the lungs of *Scnn1b*-Tg mice or wild-type littermates at a plasmid concentration of 0.5 mg/ml unless specified otherwise. One week after the administration, the lungs were harvested and homogenized to quantify the luciferase activity using conventional luciferase assay as previously described [1]. Briefly, the relative light unit was recorded by 20/20n luminometer (Turner Biosystems, Sunnyvale, CA) and subsequently normalized by the total protein concentration measured by BCA assay (ThermoFisher). For the double dose studies, animals received two consecutive doses of intratracheal DNA-MPP at an interval of one week and lung tissues were harvested one week after the second dose for homogenate-based luciferase assay.

Collection and analysis of BAL fluid and mucus plugs

We euthanized mice with deep isoflurane anesthesia and carefully harvested lungs without damaging the tissues to avoid BAL fluid leakage. Subsequently, a narrow micropipet tip connected to a 1 ml syringe was inserted into the trachea and lavage was commenced with 1 ml of PBS, followed by twice more with 1 ml each to collect an overall volume of 3 ml. Cells were then pelleted by centrifugation at 300 xg for five minutes at 4 °C and the cell-free supernatant was collected for storage at 80 °C or lyophilization to be subjected to quantitative mucin analysis. The BAL cells were resuspended in 1 ml of PBS and total number of cells were counted in a blinded manner using a hemocytometer. For the isolation of mucus plugs, BAL fluid was collected in an identical manner, following by separation of cells and other invisible solid contents using a 70-μm cell strainer (Stellar Scientific, Owings Mill, MD). The lyophilized supernatant was reconstituted in 1 ml of ultrapure water for biochemical quantification of O-linked glycoproteins, which estimates the mucin concentration, employing a previously

described protocol.[6] Briefly, 0.1 M 2-cyanoacetamide solution was prepared with 0.15 M NaOH solution, and the reconstituted supernatant samples were mixed with 2-cyanoacetamide solution and incubated at 100 °C for 30 minutes. After the reaction was quenched by 0.6 M borate buffer, fluorescence intensity was measured at excitation and emission wavelengths of 340 and 420 nm, respectively. Standard curve was generated by measuring the serially diluted solution of mucin from bovine submaxillary gland (Sigma Aldrich).

Histological staining

For hematoxylin and eosin (H&E) staining, lung tissues were harvested and fixed with 4% paraformaldehyde overnight at 4 °C. Fixed lung tissues were then embedded in paraffin and sliced at 10-μm thickness, using Leica Microtomes Biosystems RM2245 (Leica Camera, Wetzlar, Germany). After hydration, the lung slides were stained using H&E staining kit (ab245880, Abcam, Cambridge, MA) as per manufacturer's protocol, and observed with an Inverted microscope at 20x magnification. For Alcian blue/Periodic acid-Schiff (Ab-PAS) staining, lung tissues were harvested and fixed with absolute methanol overnight at 4 °C. We then slices the lung tissues as described above and the lung slides, following hydration, were stained using Ab-PAS staining kit (ab245876, Abcam) as per manufacturer's protocol and observed by Inverted microscope at 40x magnification.

***In vitro* validation of a plasmid-encoding shENaC**

We treated M-1 mouse kidney epithelial cells and NIH 3T3 mouse fibroblasts with plasmids encoding shENaC packaged in Lipofectamine 3000 (prepared at a plasmid to Lipofectamine 3000 ratio of 1:3) at varying plasmid concentrations. Three days after the treatment, we processed cells to conduct western blot analysis. Specifically, 30 μg of protein from untreated and treated cells was loaded into the wells of Novex Tris-Glycine gel (ThermoFisher). After running the gel at 120 V for 90 minutes, protein was transferred to nitrocellulose membranes and blocked with bovine serum albumin for an hour, followed by an overnight incubation with primary antibody against mouse α-subunit of ENaC (PA1-920A, TermnoFisher) at 4 °C. The membranes were washed three times with PBS and then incubated with secondary antibody (Abcam) for an hour at room temperature. Membranes were washed with PBS three times, and ECL substrate (Promega, Madison, WI) was applied for visualization of protein bands via a Chemi-Doc imaging system (Bio-RAD).

Hypertonic saline treatment

We first prepared 7 % NaCl solution and sterile-filtered with a 0.22 μm syringe filter. After anesthesia under 2 % isoflurane, *Scnn1b*-Tg intratracheally received either 7% NaCl at 1 μl/g body weight or an equal volume of ultrapure water three times per day for one week. One day after the last treatment with 7% NaCl solution, DNA-MPP carrying plasmids encoding either luciferase or shENaC were administered into the mouse lungs using a microsprayer. One week after the treatment with DNA-MPP, lung tissues or BAL fluids were harvested for further analysis.

Evaluation of ENaC expression in the lung

We determined the level of ENaC expression in the lungs of *Scnn1b*-Tg mice with or without inhaled treatment with a single or dual dose(s) of shENaC-MPP at a plasmid concentration of 0.5 mg/ml. One week after the final administration, lung tissue were harvested from animals and

homogenates were prepared for each mouse. Subsequently, western blot analysis was conducted as described above, but using 40 µg of protein from each sample.

ALI culture studies – *in vitro* transgene expression

The University of Alabama at Birmingham Institutional Review Board approved the use of primary CF HBE cells harvested from an F508del homozygous CF patient for the studies described below. Briefly, primary CF HBE cells were first expanded by co-culture with irradiated fibroblasts (R&D Systems, Minneapolis, MN), followed by conditional reprogramming via media containing a rho-associated protein kinase inhibitor. Primary CF HBE cells were grown in 0.33 cm^2 polyester membrane Transwell® inserts (Corning, Corning, NY) coated with NIH 3T3 fibroblast conditioned media until reaching 100% confluency. Primary CF HBE cells were then maintained in differentiation media for at least 4 - 6 weeks to be terminally differentiated. An 8 µL suspension of DNA-CP or DNA-MPP at a plasmid concentration of 0.1 mg/ml was administered to the apical surface of each ALI culture. Two days after the administration, the Transwell® inserts were cut out using a micro-scalpel, placed onto a glass slide and sealed with a coverslip for microscopic observation. To avoid artifacts potentially introduced due to variation in focus among images, we used automated focusing, available in Zeiss Zen software, of DAPI cell nuclear staining and captured all images at this identical focal plane. Four or more randomly selected image fields were taken from each insert to be quantitatively analyzed. The percentage of GFP coverage was defined as the GFP-positive area divided by the total cell-covered surface area, and the total GFP intensity was quantified as the average of total pixel intensity units for each image field, in a blinded manner. Prior to quantitative analysis, fluorescence of all the images taken from ALI cultures treated with DNA-CP or DNA-MPP were normalized by fluorescence of untreated control cultures to eliminate the contribution of autofluorescence.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). Two or multiple comparisons were conducted by employing two-sided Student's *t*-test or one-way analysis of variance (ANOVA) with appropriate post-hoc analyses, respectively. Differences were considered to be statistically significant at a level of $p < 0.05$.

References

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Table S1. Physicochemical characterization of DNA-CP and DNA-MPP carrying luciferase-, GFP-, and shENaC-encoding plasmids.

DNA-loaded nanoparticle	Plasmid payload (coded protein)	Hydrodynamic diameter (nm)	Polydipersity index	Z-potential (mV)
DNA-CP	luciferase	110 ± 6	0.19	28 ± 3
	GFP	104 ± 4	0.18	27 ± 4
	shENaC	105 ± 3	0.16	29 ± 3
DNA-MPP	luciferase	55 ± 3	0.11	1.0 ± 0.3
	GFP	58 ± 4	0.13	0.9 ± 0.4
	shENaC	57 ± 4	0.15	0.8 ± 0.2

Table S2. Physicochemical characterization of DNA-CP and DNA-MPP carrying luciferase-encoding plasmids before and after spraying via an aerosol-generating microsprayer

DNA-loaded nanoparticle		Hydrodynamic diameter (nm)	Polydipersity index	Z-potential (mV)
DNA-CP	Fresh	107 ± 3	0.15	30 ± 4
	Microspray	109 ± 5	0.18	29 ± 5
DNA-MPP	Fresh	54 ± 1	0.12	0.8 ± 0.1
	Microspray	53 ± 2	0.13	0.7 ± 0.3

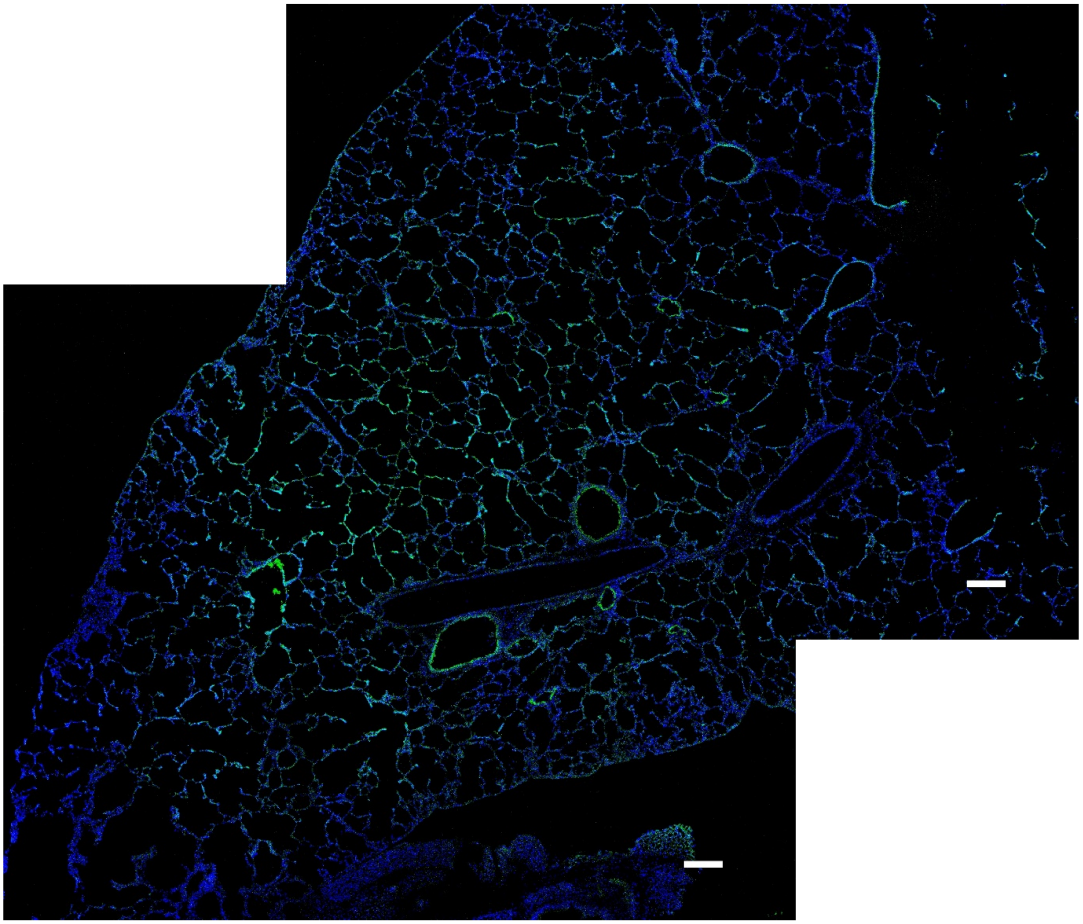

Figure S1. Representative confocal image of *Scnn1b*-Tg mouse lung treated with DNA-MPP carrying GFP-encoding plasmids. Widespread and uniform GFP transgene expression (green) was observed through a whole left lobe of lung. Cell nuclei are stained with DAPI (blue). Scale bar = 500 μm

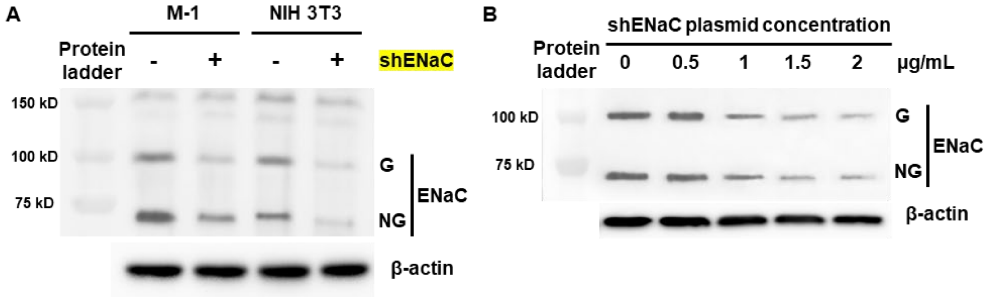

Figure S2. *In vitro* validation of a plasmid encoding shRNA against ENaC. (A) ENaC expression was suppressed in M-1 mouse kidney epithelial cells and NIH 3T3 mouse fibroblasts through the treatment with shENaC-encoding plasmids packaged in Lipofectamine 3000 at 1:3 w/w ratio as per the manufacturer's protocol. **(B)** Dose-dependent suppression of ENaC was observed in M-1 mouse kidney epithelial cells treated with shENaC-encoding plasmid/Lipofectamie 3000. G: glycosylated; NG: non-glycosylated.

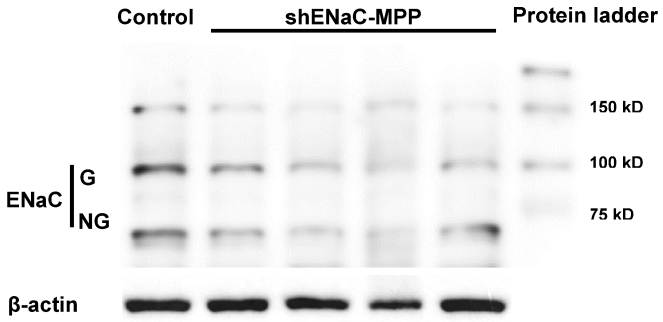

Figure S3. Western blot image demonstrating downregulation of ENaC in the lungs of S*cnn1b*-Tg mice by a single intratracheal dose of shENaC-MPP. G: glycosylated; NG: non-glycosylated.

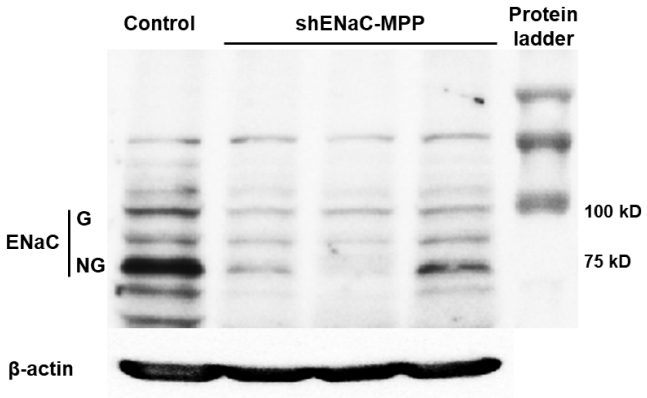

Figure S4. Western blot demonstrating downregulation of ENaC in the lungs of *Scnn1b*-Tg mice received repeated intratracheal administration (i.e. two doses) of shENaC-MPP. G: glycosylated; NG: non-glycosylated.

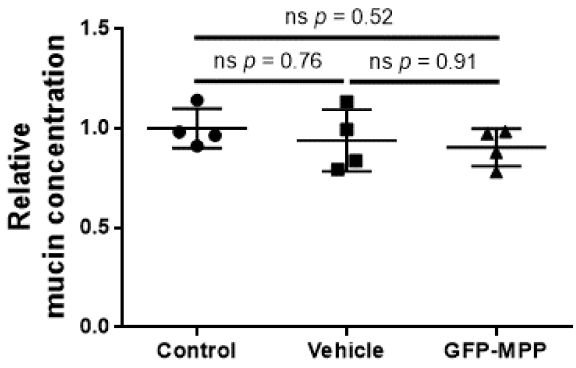

Figure S5. Quantification of O-linked glycoproteins (*i.e.*, mucin) in the lungs of *Scnn1b*-Tg mice that were untreated, vehicle-treated or treated with DNA-MPP carrying GFP-expressing plamids (GFP-MPP). The differences are not statistically significant as indicated.