Supporting Information

Guanylurea-functionalized conjugated polymer enables RNA interference in ex vivo human airway epithelium

Materials and Methods. Chemicals and solvents were purchased from Fisher Scientific and used without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Cambridge, MA). All solutions were prepared using deionized (DI) water (~18MΩ) from water purification system (Ultra Purelab system, ELGA/Siemens). The number average molecular weight ($M_n$), weight average molecular weight ($M_w$), and polydispersity index (PDI = $M_w/M_n$) of CPs were determined by gel permeation chromatography (GPC) against polystyrene standards using a Shimadzu high performance liquid chromatography (HPLC) system fitted with PLgel 5µm MIXED-D columns and SPD-20A ultraviolet-visible (UV-vis) detector at a flow rate of 1.0 mL/min. Samples for GPC, small amounts (~100 µL) of polymer in dimethylformamide (DMF) or dichloromethane (DCM) was diluted with 1 mL of HPLC grade THF and then filtered through a 0.45 µM polytetrafluoroethylene (PTFE) syringe filter prior injection. UV-vis spectra were recorded using a Varian Cary 50 Bio spectrophotometer. Fluorescence spectra were obtained using a FluoroLog-3 Spectrofluorometer (Jobin Yvon/Horiba). 9,10-diphenylanthracene (QY = 0.9) in cyclohexane was used as a fluorescence standard. Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer Spectrum 100 FTIR Spectrometer. Fine polymer powders were directly mounted on an attenuated total reflection (ATR) cell of the spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Avance Bruker NMR spectrometer. Chemical shifts were reported in parts per million (ppm) for $^1$H NMR on the $\delta$ scale based on the middle peak ($\delta = 2.50$ ppm) of the dimethylsulfoxide (DMSO-d$_6$) solvent as an internal standard. AFM samples were prepared by dropping a minute amount of solution onto silicon substrates and storing them inside a vacuum chamber until dry. The topography of polyplexes was scanned using
Bruker Multimode AFM Tapping module. The analysis of topography images was conducted using NanoScope Analysis 1.5 software.

**Synthesis of monomer A.** Guanidine-containing aryldiiodide monomer A was synthesized according to our published procedure.\(^\text{1}\)

\[\text{I} \quad \text{I} \quad \text{R} \quad \text{OR} \quad \underset{\text{Pd}(\text{PPh}_3)_2\text{Cl}_2, \text{CuI, THF}}{\underset{\text{TMSA, r.t.}}{\rightarrow}} \text{TMS} \quad \underset{\text{K}_2\text{CO}_3, \text{MeOH}}{\underset{\text{r.t., 30 min}}{\rightarrow}} \text{OR} \quad \text{A} \quad \text{B} \]

\[\text{R} = \text{O} \quad \text{H} \quad \text{NHBOc} \quad \text{NBoc} \]

**Synthesis of monomer B.** Compound A (2.00 g, 1.96 mmol), trimethylsilylacetylene (0.77 g, 7.84 mmol), \(\text{PdCl}_2(\text{PPh}_3)_2\) (137.6 mg, 0.20 mmol), and CuI (18.6 mg, 0.10 mmol) were combined in a flask. The flask was evacuated and filled with \(\text{N}_2\) three times. A mixed solution of tetrahydrofuran (THF) and diisopropylamine (DIPA) (4:1, v/v) was degassed with \(\text{N}_2\) for 10 min, and then 50 mL was transferred to the flask via a cannula. The reaction was stirred at room temp for 3 h equipped with a \(\text{N}_2\) balloon. The yellow reaction mixture was filtered to remove insoluble precipitates and then THF was distilled out in vacuo. The reaction mixture was dissolved in DCM and washed with 1M NH\(_4\)Cl followed by a brine solution. Column chromatography under 30% ethyl acetate in hexane yielded a final product of a white solid (1.22 g, 65% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\), \(\delta\)) : 11.46 (s, 1H), 8.67 (s, 1H), 7.21 (s, 1H), 4.1 (t, 2H), 3.88 (t, 2H), 3.78 (t, 2H), 3.66 (q, 2H), 1.50 (s, 9H), 1.45 (s, 9H), 0.10 (s, 18H).

In a flask, trimethylsilyl (TMS)-protected compound (1.00 g, 1.04 mmol), and potassium carbonate (0.36 g, 2.60 mmol) were mixed in methanol (40 mL). The flask was then stirred at room temp. for 20 min. Upon confirmation of TMS deprotection by TLC, the solvent was dried in vacuo. The reaction mixture was then purified by short-path column chromatography under 35% ethyl acetate in hexane, yielding a
yellowish solid (0.51 g, 60% yield). $^1$H NMR (400 MHz, CDCl$_3$, $\delta$): 11.45 (s, 1H), 8.67 (s, 1H), 6.98 (s, 1H), 4.15 (t, 2H), 3.86 (t, 2H), 3.75 (t, 2H), 3.65 (q, 2H), 3.35 (s, 1H; CH), 1.50 (s, 9H), 1.45 (s, 9H). $^{13}$C NMR (400 MHz, CDCl$_3$, $\delta$): 156.40, 154.21, 153.13, 118.34, 113.80, 83.20, 83.10, 79.60, 79.40, 70.00, 69.80, 69.40, 40.90, 28.40, 28.20; FT-IR (neat): $\nu$ = 3331, 3281, 2975, 2931, 1720, 1636, 1613, 1568, 1496, 1410, 1320, 1223, 1129, 1050 cm$^{-1}$; HRMS (ESI, m/z): [M + H $\text{C}_{40}\text{H}_{60}\text{N}_{6}\text{O}_{12}$]$: +$ calculated 817.4342; found 817.4365.

**Synthesis of Boc-protected Poly 1.** A Schlenk flask was filled with monomer A (50.0 mg, 0.04 mmol), B (40.0 mg, 0.04 mmol), PdCl$_2$(PPh$_3$)$_2$ (3.43 mg, 0.005 mmol), and CuI (0.47 mg, 0.003 mmol). The Schlenk flask was evacuated and filled with N$_2$ three times. A mixed solution of THF and DIPA (4:1, v/v) was degassed using N$_2$ flow, and 2.00 mL of the solvent was transferred to the reaction flask.

The reaction flask was stirred at room temperature for 16 h. The solution was then filtered through a glass wool filled glass pipette and transferred dropwise to methanol, resulting in precipitation. The supernatant was decanted, the precipitates were re-dissolved in DCM (0.50 mL), and the purification in methanol was repeated three times. The overall yield was 65% (26.9 mg). $^1$H NMR (400 MHz, CDCl$_3$, $\delta$): 11.46 (s, 1H), 8.62 (s, 1H), 7.05 (s, 1H), 4.24 (s, 2H), 3.91 (s, 2H), 3.74 (s, 2H), 3.62 (s, 2H), 1.48 (s, 9H), 1.45 (s, 9H); FT-IR (neat): $\nu$ = 3330, 3132, 2978, 2931, 1721, 1615, 1614, 1567, 1504, 1412, 1365, 1320, 1281, 1250, 1131, 1049 cm$^{-1}$; GPC: $M_n$ = 13,500 g/mol, $M_w$ = 18,000 g/mol, PDI = 1.30; UV-Vis (THF): $\lambda_{\text{max}}$ = 442 nm; Fluorescence (THF, Excitation = 442 nm) $\lambda_{\text{max}}$ = 469 nm.
Figure S1. $^1$H NMR of Boc-protected guanidine-functionalized Poly 1.

Figure S2. FT-IR of Boc-protected Guanidine-functionalized Poly 1.
**Synthesis of Boc-protected Poly 2.** A Schlenk flask was charged with the Boc-protected Poly 1 (10.0 mg, 0.012 mmol) and aminoethoxyethanol (3.10 mg, 0.03 mmol). The Schlenk flask was evacuated and filled with N\(_2\) three times. Degassed THF (1.5 mL) was transferred to the Schlenk flask via a cannula. The reaction was stirred at 80 °C for 16 h. A viscous polymer solution was filtered by a glass wool filled glass pipette and precipitated in diethyl ether and methanol. The final polymer was a yellow gel (8.20 mg with 79% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\), δ): 12.05 (s, 1H), 8.27 (s, 1H), 7.04 (s, 1H), 6.03 (s, 1H), 4.22 (s, 2H), 3.90 (s, 2H), 3.72 (s, 5H), 3.53 (s, 8H), 3.36 (s, 2H), 1.42 (s, 7H); FT-IR (neat): ν = 3320, 2930, 2870, 1716, 1633, 1595, 1507, 1454, 1411, 1348, 1312, 1277 cm\(^{-1}\); GPC: \(M_n = 13,800\) g/mol, \(M_w = 19,600\) g/mol; PDI = 1.40. UV-Vis (THF) \(\lambda_{\text{max}} = 435\) nm, Fluorescence (THF, Excitation = 435 nm) \(\lambda_{\text{max}} = 465\) nm.

**Figure S3.** \(^1\)H NMR of Boc-protected Poly 2.
Synthesis of Boc-protected Poly HA. A Schlenk flask was charged with Poly 1 (10 mg, 0.012 mmol) and Hexylamine (4.78 mg, 0.05 mmol). The Schlenk flask was evacuated and filled with N₂ (3x). A degassed tetrahydrofuran (THF) (1.5 mL) was transferred to the Schlenk flask via a cannula. The reaction was stirred at 80 °C for 16 h. A viscous polymer solution purified by glass wool filled glass pipette and precipitated in diethyl ether and methanol. The final polymer was a yellow gel (6.33 mg with 62% yield). ¹H NMR (400 MHz), CDCl₃, δ: 12.2 (s, 1H), 8.23 (s, 1H), 7.05 (s, 1H), 5.54 (s, 1H), 4.22 (s, 2H), 3.90 (s, 2H), 3.74 (s, 2H), 3.50 (s, 2H), 3.49 (s, 2H), 3.13 (s, 2H), 1.43 (s, 9H). FT-IR (neat): 3350.6, 3023.9, 2955.5, 2922.5, 2854.8, 1716.9, 1685.9, 1634.4, 1597.2, 1504.8, 1489.9, 1367.1, 1320.9, 1280.8. GPC: $M_n = 11,200$ g/mol, $M_w = 16,800$ g/mol, PDI = 1.50. UV-Vis (THF) $\lambda_{\text{max}} = 416$ nm, Fluo $\lambda_{\text{max}} = 469$ nm. QY = 36.
Synthesis of Boc-protected Poly mPEG7. A solution of Poly 1 (30 mg, 0.035 mmol) in 1mL THF was prepared. 45mg (0.141 mmol) of mPEG7-NH₂ (Sigma-Aldrich) was added to the solution and stirred overnight at 73°C. Polymer solution was then filtered followed by three times precipitation against
ether. $^1$H NMR (600 MHz, CDCl$_3$, $\delta$): 12.07 (s, 1H), 8.24 (s, 1H), 7.05 (s, 1H), 5.68 (s, 1H), 4.22 (s, 2H), 3.88 (s, 2H), 3.62 (30H), 3.52 (s, 5H), 3.35 (s, 5H), 1.41 (s, 7H);

Figure S7. $^1$HNMR of Boc-protected Poly mPEG7.

**General procedure for Boc-deprotection of Poly 1, 2, HA, and mPEG7.** In a vial, a solution of Boc-protected polymer in DCM (1.00 mL) was treated with trifluoroacetic acid (TFA) at room temperature for 48 h. The solvent was removed under reduced pressure, and the crude material was dissolved in a minimum amount of dimethylformamide (DMF) to have a clear homogeneous solution. The polymer solution in DMF was transferred to diethyl ether, resulting in yellowish fiber-like precipitates that were collected by decantation. The polymer was dissolved in DMF and then re-precipitated in ethyl acetate. This process was repeated twice, and the final Boc-deprotected polymer was collected by decantation followed by vacuum dry.
Poly 1. Following the general procedure described above, the final deprotected polymer was a yellow gel (74.6 % yield). $^1$H NMR (400 MHz, DMSO-d$_6$, δ): 8.13-6.9 (m, 12H), 4.40 (br, 2H), 4.02 (br s, 2H), 3.72-3.61 (m, 4H); FT-IR (neat): $\nu$ = 3327, 2979, 2933, 1722, 1637, 1613 cm$^{-1}$; UV-Vis (DMSO) $\lambda_{\text{max}}$ = 434 nm,

Fluorescence (DMSO, Excitation = 434 nm) $\lambda_{\text{max}}$ = 490 nm; QY = 0.08

Figure S8. $^1$H NMR of Poly 1.
**Figure S9.** FT-IR of Poly 1.

**Poly 2.** Following the general procedure described above, the final deprotected polymer was a yellow gel (79% yield). $^1$H NMR (400 MHz, DMSO-d$_6$, $\delta$): 9.21 (s, 1H), 8.51 (br s, 2H), 7.90-7.60 (m, 3H), 4.47-3.80 (m, 11H), 3.24 (br s, 4H); FT-IR (neat): $\nu = 3267, 2972, 1788, 1670, 1547, 1447, 1347, 1199$ cm$^{-1}$; UV-Vis (DMSO) $\lambda_{max} = 434$ nm, Fluorescence (DMSO, Excitation = 434 nm) $\lambda_{max} = 494$ nm; QY = 0.20

**Figure S10.** $^1$H NMR of Poly 2.
**Figure S11.** FT-IR of Poly 2.

**Figure S12.** $^1$H NMR of Poly HA.

**Poly mPEG7.** $^1$H NMR (600 MHz, DMSO-d$_6$, δ): 9.29 (s, 1H), 8.53 (br s, 1H), 8.20-7.00 (m, 3H), 4.38 (br s, 2H), 4.03 (br s, 2H), 3.90-3.60 (m, 4H), 3.47 (s, 30H), 3.21 (s, 4H). FT-IR (neat): $\nu$ = 3614, 2937, 2249, 1691,
1617, 1360, 1055, 1004 cm\(^{-1}\). UV-Vis (DMSO) \(\lambda_{\text{max}} = 421\) nm, Fluorescence (DMSO, Excitation = 430 nm) \(\lambda_{\text{max}} = 487\) nm.

Figure S13. 1H NMR of Poly mPEG7.
Figure S14. FTIR of Poly mPEG7.
A

B

C

Poly-1

Poly-1

Poly-Z

Poly-Z
Figure S15. (A) UV absorbance (left) and emission (right) spectra of Poly 1 and Poly 2 in DMSO and water, respectively. (B) Absorption and emission spectra of Boc-protected Poly HA. (C) Absorption and emission spectra of Poly mPEG7 in DMSO.

Characterization of Polyplexes. The HD and zeta potential of Poly 1 and Poly 2 were determined using Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering (DLS), respectively. For NTA and DLS, all cuvette, pipette, and pipette tips were autoclaved. Additionally, the working area was cleaned with 70% ethanol to avoid any cross-contamination. In that measurement, stock polymer samples were prepared at a concentration of 100 μM in DMSO solvent. 10 μL of a stock polymer solution in DMSO was added to 90 μL of RNase-free water. The polymer solution was mixed with 900 μL of siRNA (11.11 nM) in water (final polymer and siRNA concentrations are 1 μM and 10 nM, respectively). The mixture of polymer and siRNA solution was gently pipetted, and 1 mL sample solution was then injected to the NTA chamber, and images of scattering particles in the sample were collected for 90 seconds. The software identified each individual particle and tracked its motion throughout the duration of the recorded video. The measured particle displacement is a function of Brownian motion, which is related to the particle size through the Stokes-Einstein equation. The final data was collected under the detection threshold at 4, to obtain the acceptable data meeting the concentration requirements. All measurements were performed in triplicate at 25 °C using a temperature controller. The values were averaged from three independent measurements.
Zeta potentials of Poly 1/siRNA and Poly 2/siRNA complexes were measured using Zetasizer Nano-ZS (Zen 3600, Malvern Instruments Ltd.). The viscosity and refractive index of water were used for estimation of relative zeta potential difference among the samples. Stock polymer samples were prepared at a concentration of 1000 μM in DMSO. 10 μL of stock polymer solution was dissolved in 90 μL RNase-free water. Then the polymer solution in water was transferred to 900 μL of siRNA containing water (siRNA concentration 11.11 nM) and the solution was mixed by pipetting. The final polymer and siRNA concentrations were 10 μM and 100 nM, respectively. All measurements were performed in triplicates at 25 °C and the average values were reported.

Cell culture. Primary human bronchial epithelial cells were isolated and re-differentiated at the air-liquid interface (ALI) cultures as described by Fulcher and Randall\textsuperscript{2,3} adapted by us.\textsuperscript{4,5} Cells were obtained from properly consented donors whose lungs were not suitable for transplantation for the causes unrelated to airway complications and supplied by the University of Miami Life Alliance Organ Recovery Agency. Since the material was obtained from deceased individuals with minor, de-identified information, its use does not constitute human subjects research as defined by CFR 46.102. A signed and well-documented consent of each individual or legal healthcare proxy for the donation of lungs for research purpose is on file with the Life Alliance Organ Recovery Organization allows research purpose of this material. Unless otherwise mentioned, experiments used cells from non-smokers to not confound the findings in unknown ways. These primary cultures undergo mucociliary differentiation at the ALI reproducing both the in vivo morphology and key physiologic processes to recapitulate the native bronchial epithelium ex vivo.\textsuperscript{2,3}

The immortalized normal human bronchial epithelial cell line BEAS-2B (ATCC CRL-9609) was purchased from the American Type Culture Collection (Manassas, VA, USA). BEAS-2B cells were cultured in BioLite 75 cm\textsuperscript{2} flasks (Cat. #130190, Thermo Scientific) containing Bronchial Epithelial Cell Growth Medium (BEGM). BEGM media was supplemented with 0.1% (v/v) human recombinant epidermal growth factor,
0.1% (v/v) insulin, 0.1% (v/v) hydrocortisone, 0.1% (v/v) ethanolamine, 0.1% phosphoryl ethanolamine, 0.1% (v/v) retinoic acid, 0.1% (v/v) epinephrine, 0.24% (v/v) transferrin, 1% (v/v) penicillin/streptomycin and 0.1% (v/v) bovine pituitary extract as published by Fulcher et al. The cells were cultured in 95% air and 5% CO2 at 37 °C and maintained free of mycoplasma contamination.

**Cell viability assay.** BEAS-2B cells (~15,000 cells per well) in 200 μL of a complete medium, were seeded into a 96-well plate and allowed to attach for one day at 37 °C under humidified atmosphere of 5% CO2 / 95% air. Final concentrations of 40 µM, 20 µM, 10 µM, 5 µM, and 1 µM of CPs were added into the complete media by dilution with CPs stock solutions. After addition of CPs, cells were incubated for another 18 h. The cells were treated with 10 µL of methylthiazole tetrazolium (MTT) (5 mg/mL in PBS, CALBIOCHEM, Germany) and incubated for 4 h at 37°C. Then, 200 µL of medium was removed gently by using a pipette and then 100 µL of biological grade DMSO (Sigma Aldrich, St. Louis, MO, USA) was added to solubilize the purple formazan crystals formed by proliferating cells. Absorbance was measured by a microplate well reader (infinite M1000 PRO, TECAN, Switzerland) at 570 nm. Relative cell viability (%) as a function of CPs concentration was expressed as the percentage relative to the untreated control cells. All measurements were performed in triplicate, and the standard deviation was included in the error bar.
**Figure S16.** Cell viability inhibition by Poly 1 and Poly 2.

**Gel retardation assay.** The siRNA binding capabilities of Poly 1 and Poly 2, respectively, were examined by a gel retardation assay. 10 μL of Ambion Negative Control siRNA (400 nM) (Thermo Fisher Scientific) was mixed with 10 μL of the polymers with different concentrations. Samples were gently vortexed and kept for 30 min at room temperature. Then, polyplexes solutions (10 μL) were mixed with 10 μL of 2xRNA loading buffer (Thermo Fisher Scientific). The polyplexes solutions (20 μL) were loaded into 2% agarose gel and run in 1X TBE buffer at 80V for 30 min. Free siRNA bands were visualized using 0.5 μg/mL ethidium bromide solution. The bands were visualized by using the Biorad Universal Hood II Gel Doc System (Bio-Rad Laboratories, USA) and the density values are normalized to free siRNA.
Figure S17. siRNA binding efficiency of Polymers measured using gel retardation assay. Bottom image is a gel image randomly selected from three independent assays. The band intensity of free siRNA (N/P ratio of 0) was used to normalize the relative amounts of unbound siRNA at different N/P ratios.

Mucopenetration measurement. A mucus mimic was prepared by dissolving Mucin Type III (Sigma-Aldrich) in ultrapure water at the concentration of 10 mg/mL, followed by heating at 37 °C overnight. Transwell inserts (polyester 0.4 μm pores, 12 mm diameter, Corning) were used to examine the mucopenetration behavior of CP/siGLO complexes with respect to incubation time. Briefly, 1 mL of ultrapure water was added to the receiver compartment of a 12 well plate well and 100 μL of mucus mimic solution was added to the donor compartment on the membrane. This was allowed to settle for 30 minutes to afford an approximately 880 μL thick mucus layer. This mucus layer is much thicker than the physiological relevant thickness, however, due to the small volumes that would be required to be used, this model was chosen to demonstrate the mucopenetration behavior. To this settled mucus layer, 100 μL of sample solution was carefully added and the plate was incubated at 37 °C to allow for migration of the complexes through the mucus layer. At various time intervals, 100 μL of solution from the receiver compartment was taken from each well and siGLO concentration was measured using a Licor Synergy plate reader (ex: 530; em: 580).

Immunocytochemistry for cilia to determine differentiation. NHBE cells were allowed to re-differentiate for 21 days at the ALI on transwell filters. Re-differentiation was determined by staining for ciliation as described by us earlier. Briefly, cells were fixed in 4 % paraformaldehyde in PBS, pH 7.4 for 15 min and permeabilized with 1 % Triton X-100 in PBS for 20 min at room temperature. After permeabilization, cells were washed with PBS and then blocked with 3 % BSA in PBS for 1 h at room temperature. Cells were treated with the primary antibody [mouse anti human acetylated α-tubulin (Sigma Cat. #T6793; 1:1000)] in blocking solution and incubated overnight at 4 °C. Cells were washed three times and then incubated with Alexa 647 anti mouse IgG for 45 min. Cells were washed three times with blocking solution and
counterstained with 4,6-diamidine-2-phenylindole (DAPI, KPL) to label nuclei for 10 min. Transwell insert filters were excised and placed directly on the slide and images were acquired on visualized using a Zeiss fluorescence microscope with high-resolution Axiocam 506 mono microscope camera (Zeiss, Germany). Cilia appear green at the apical side of the NHBE cultures with nuclei stained in blue.

**Ussing chamber method to determine apical CFTR activity.** Ussing chamber electrophysiology was used to confirm re-differentiation and polarization as reported by us earlier. CFTR is located at the apical side (mucosal) of the airway epithelium. Briefly, NHBE cultures were re-differentiated at the ALI. Following re-differentiation for 21 days, the snap wells were removed from supports and mounted in Ussing chambers. The short circuit current was measured and allowed to stabilize followed by addition of amiloride (10 µM) added apically to eliminate epithelial sodium channel (ENaC) influences. CFTR activation was affected by addition of albuterol (10 µM) and change in short circuit current ($\Delta I_{sc}$) was determined. CFTR specificity was confirmed by addition of CFTR inhibitor CFTR inh 172 (20 µM) and the decrease in $\Delta I_{sc}$ was recorded.

![Figure S18](image)

**Figure S18.** The physiological evidences of well-differentiated NHBE cells. a) Immunocytochemical staining of well-differentiated NHBE cells. Cilia appear as green at the apical side of the NHBE cultures with nuclei stained in blue. Scale bar: 40 µm. b) Upon addition of a CFTR activator (albuterol) followed by an inhibitor (CFTR inh 172), a dramatic apical current change in NHBE cells mounted in a Ussing chamber was monitored, supporting that the cultured NHBE cells exhibit the characteristic membrane polarization of epithelium. Amiloride was used to block the epithelial sodium channel.
Figure S19. Confocal microscopic images of BEAS-2B cells incubated with CP/siGLO polyplexes for 1 h visualize CP-mediated siRNA delivery. Green dots were observed from CPs located in the cytosol (a and d). Red signals from siGLO were observed from cells treated with CPs (b and e), while the intensity of siGLO was much stronger from cells treated with Poly 2/siGLO (e). While the combined image of Poly 1 shows that CP and siGLO are mainly overlapped (c), some portions of siGLO were released from Poly 2 (f). Scale bar: 20 μm.

Confocal microscopic images. BEAS-2B cells (~0.5x10^6/well) were seeded into a 12-well plate with glass coverslip one day prior to CP treatment, and then cultured in a complete media for 24 h under 5% CO₂ at 37 °C. Cells were washed three times with 1× PBS after removing the media. The polyplex formed by mixing 10 μM CPs and siGLO (100 nM) was added to cells and then incubated for 48 h. After 48 h incubation, cells were washed three times with 1× PBS and fixed with 4% PFA for 10 m. Cells were then washed three times with 1× PBS and coverslips were mounted on microscope slides using a 1:1
**Figure S20.** Fluorescence microscopic image of primary NHBE cells treated Poly 1/siGLO (a-c), Poly 2/siGLO (d-f), and Lipofectamine/siGLO (g-i), respectively. Significant amounts of Poly 2 (d) and siGLO (e) were observed, while background signals were seen from the cells treated with Poly 1 and Lipofectamine, respectively. Scale bar: 20 μm.

glycerol/PBS mounting medium. Fluorescent images of the cells were obtained using an Olympus Fluorview FV1200 confocal microscope (Melville, NY USA) equipped with a bandpass filter for green (513-556 nm) and a 60X oil immersion lens (NA 1.35, n = 1.519 immersion oil). Image J software (Version 1.50b, U.S. National Institute of Health, Bethesda, Maryland, USA) was used to process the image.

**Lipofectamine RNAiMAX-mediated transfection of siHDAC in BEAS-2B cells.** High-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Cat. #4368814). Taqman Fast Advanced Master Mix was purchased from Life Technologies (Cat. #4444557). Lipofectamine® RNAiMAX Transfection Reagent and Opti-MEM™ Reduced-Serum Medium were purchased from Thermo Fisher Scientific (cat. Nos. 13778150 and 31985062). BEAS-2B cells were plated on collagen coated tissue culture plates at a density of 0.6x10^6. Twenty-four hours following plating, cells were transfected with siHDAC complexed with Lipofectamine RNAiMax in Opti-MEM medium according to manufacturer’s instructions using different concentrations of the siRNA (i.e., 25, 50, 75, and 100 nM). BEAS-2B cells treated with equivalent amounts of lipofectamine RNAiMAX in Opti-MEM was used as transfection control. The mixture was vortexed and incubated at room temperature for 20 m before adding to the cells. After eight-hour post-transfection, experiment was terminated, and total RNA was isolated and analyzed by quantitative RT-PCR.
**Polymer-mediated siHDAC knockdown in BEAS-2B cells.** The polyplex solutions were freshly prepared prior to transfection experiments. One day after plating BEAS-2B cells (0.6x10^6) in a 12-wells plate, cells were transfected by adding polyplex solutions. 5 mM polymer stock solution was diluted to 20µM in 50 µl of RNase and DNase free water, and then mixed with various amounts of siHDAC (i.e., 25, 50, and 100 nM). Polyplex solution was vortexed for 30 min. Cells were incubated with polyplex for 48 h. The total RNA was analyzed by quantitative RT-PCR.

![Figure S21. HDAC mRNA knockdown efficiency in BEAS-2B cells.](image)

**HDAC2 knockdown experiment in NHBE cells.** The polyplex solutions were freshly prepared prior to transfection experiments. NHBE cultures re-differentiated at the air-liquid interface (ALI) were transfected by adding polyplex solutions using a protocol identical to that for BEAS2B cells above. Separately, another set of NHBE ALI cultures were treated with siRNA complexed with Lipofectamine RNAiMax for comparison. Experiments were allowed to proceed for 48 hours and total RNA was isolated and analyzed by quantitative RT-PCR. Total RNA was extracted from cells treated with transfection
agents after 48 h incubation using a RNeasy mini kit (Qiagen Inc. Valencia, CA). The concentration and integrity of the extracted RNA were analyzed by measurement of the OD260/280 (Synergy™ HTX Multi-Mode Microplate Reader, Winooski, VT, USA). Complementary DNA (cDNA) was reversely transcribed by using the Applied Biosystems High performance kit (Applied Biosystem, Carlsbad, CA). Reverse transcription of 2 μg of total cellular RNA was performed in a final volume of 20 μl containing 10 μl of RNA, 2 μl of 10X RT buffer, 0.8 μl of dNTP Mix (100 mM), 2.0μl of 10X RT random hexamer primers, 1.0 μl of MultiScribe™ reverse transcriptase, 1μl of RNase inhibitor, and 3.2 μl of nuclease-free water. The reverse transcription reaction was allowed to proceed using cycling parameters recommended by the manufacturer: an initial incubation at 25°C for 10 m followed by incubation at 37°C for 120 m. The reverse transcription was terminated by incubating at 85 °C for 5 sec. cDNA samples were stored at −20 °C until further use for quantitative PCR. Quantitative PCR was performed on the Bio-Rad CFX96 real-time system (BioRad, Hercules, CA, USA) using validated TaqMan probes (GAPDH, cat #Hs02758991_g1, HDAC2, cat # HS00231032-m1,) according to manufacturer recommended cycling parameters (an initial denaturation cycle of 95°C for 20 s followed by 40 cycles of 95°C/3 s and 60°C/30 s. qRT-PCR results are represented as relative quantification normalized to internal control (GAPDH).

**Flow cytometry.** Cellular entry mechanism was evaluated using a BD FACS Melody Cell Sorter under FITC channel. Fluorescent intensities of the sample cells incubated with Poly 2/siRNA complex in the presence of various pharmacological inhibitors were normalized to that of control cells only treated with the polymer/siRNA complex without an inhibitor pretreatment. BEAS-2B cells (~0.5x10⁶/well) were seeded into a 12-well plate (Cat. #12-556-005) one day before polymer/inhibitor treatments. After 24 h, cells were pre-incubated with the pharmaceutical inhibitors [chlorpromazine (10 μg/ml) for clathrin mediated endocytosis (CME); genistein (200 μM) for caveolae-mediated endocytosis (CvME); LYS294002 (3 μM) for macropinocytosis (MPC1); cytochalasin D (10 μM) for macropinocytosis (MPC2); and NaN₃ (10 mM) in the presence of 2-deoxyglucose (50 mM) for ATP depletion (i.e., nonenergy dependent)] for 30
min prior to the exposure of the polymer/siRNA complexes. 10 µM of polymer was complexed with scrambled siRNA (50 nM) and then the complexes were added to the cells pretreated with various inhibitors for 90 min. After the incubation, cells were washed with PBS for three times. Cells were harvested by using 0.25% trypsin/EDTA (Gibco #25200056) and Trypsin neutralizer solution (Cat #R002100), and then resuspended in a culture medium. After centrifugation (2000 rpm for 3 min), cells were fixed with 4% PFA for 15 min, and then resuspended in 1% PBS. Samples were stored in ice before measurements. 10,000 events per measurement were recorded within the gate of control cells based on forward and side scattering of control cells to eliminate data collection from dead cells and other artifacts. The results were reported as the mean of the distribution of cell fluorescence intensity of polymers (FL1 channel, 590-620 nm wavelength range) using three independent sample sets, and the values were normalized to control cells. Error bars are the standard deviation between these independent measurements.

**Figure S22.** Fluorescent intensity of BEAS-2B (above) cells pre-treatment with various endocytoses inhibitors followed by Poly 1 incubation.

**Reference:**


