In vivo genome editing using nuclease-encoding mRNA corrects SP-B deficiency

To the Editor:

Genome editing using a variety of different nucleases holds great potential to knock out or repair disease-causing genes. An ideal nuclease delivery vehicle is short-lived, does not integrate into the genome and can enter target cells efficiently. These requirements have not yet been achieved simultaneously by any nuclease delivery vector. We and others have used modified mRNA, which is nonintegrating and provides a transient pulse of protein expression, as an alternative to traditional viral vectors[1–5]. This approach allowed us to deliver therapeutic proteins in mouse models of surfactant protein B (SP-B) deficiency[3] and experimental asthma[4]. Here we apply nuclease-encoding, chemically modified (nec) mRNA to deliver site-specific nucleases in a well-established transgenic mouse model of SP-B deficiency[6], in which SP-B cDNA is under the control of a tetracycline-inducible promoter[7]. Administration of doxycycline drives SP-B expression at levels similar to those in wild-type mice (**Supplementary Fig. 1**), whereas cessation of doxycycline leads to phenotypic changes similar to those of the human disease, including thickened alveolar walls, heavy cellular infiltration, increased macrophages and neutrophils, interstitial edema, augmented cytokines in the lavage, a decline in lung function and fatal respiratory distress leading to death within days[8,9]. We inserted a constitutive CAG promoter

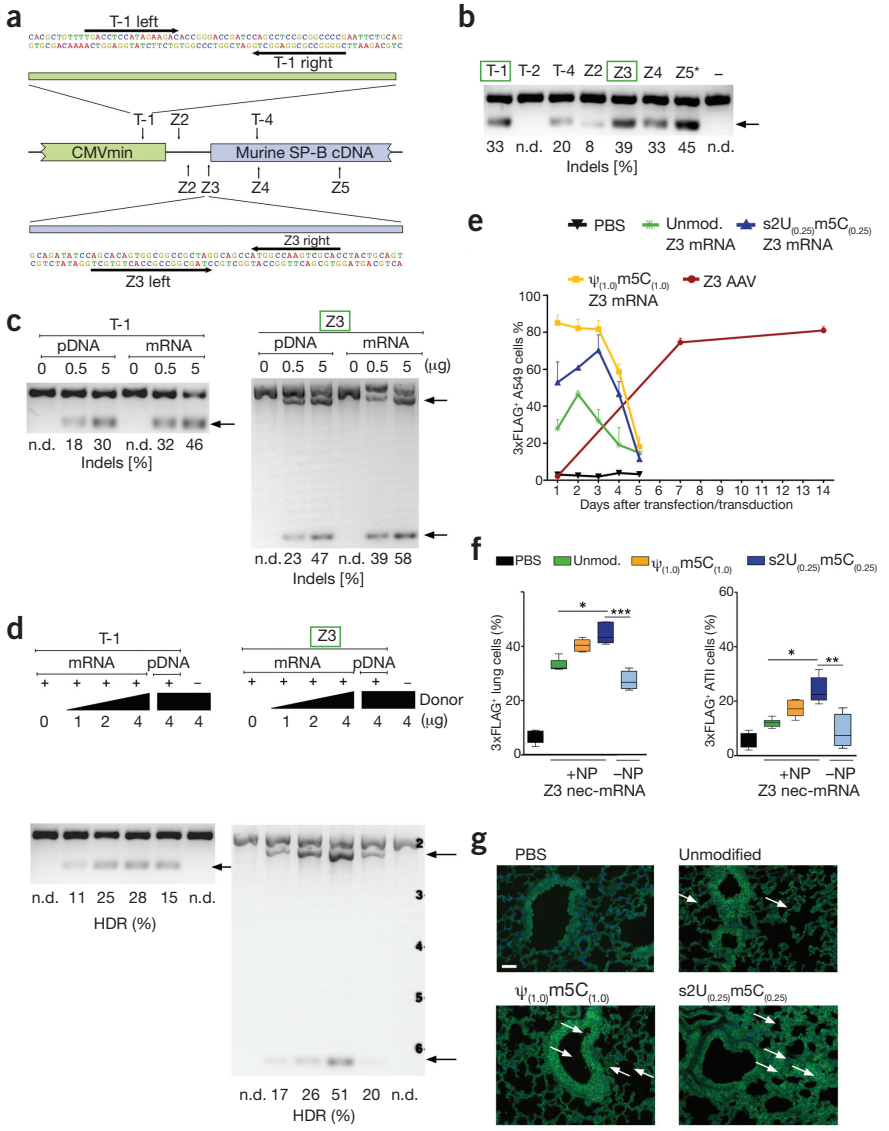

Figure 1 nec-mRNA cleaves the SP-B cassette, induces HDR *in vitro* and is expressed in lung cells *in vivo*. (**a**) T-1 and Z3 candidates relative to the transgenic SP-B cassette. CMV, cytomegalovirus. (**b**) T7 assays to determine the frequency of TALEN- and ZFN-induced indels in genomic DNA harvested 3 d after transfection (5 µg/80,000–100,000 cells). See **Supplementary Figure 21** for full blot. (**c**) T-1- and Z3-induced indels following delivery of the nucleases as either mRNA or plasmid DNA (pDNA; 0.5 or 5 µg). The preferred nuclease is marked by a green box. (**d**) Percent HDR 3 d after transfection of 5 µg T-1 or Z3 mRNA (or pDNA) with 0–4 µg donor plasmid. Arrows denote NheI-sensitive cleavage products resulting from HDR. Transgenic SP-B mice-derived fibroblasts were used for **b**–**d**; n.d., not detectable. (**e**) Time course showing kinetics and stability of 3xFLAG-tagged Z3 mRNA versus Z3 AAV in A549 cells ($n = 3$). Error bars, mean ± s.d. (**f**) Anti-3xFLAG flow cytometry shows protein expression in total lung cells and ATII cells. The light blue box presents data from the experimental setup of the dark blue box but without NPs. Boxes, medians ± interquartile ranges; whiskers, minimum and maximum; $^{*}P < 0.05$ versus unmodified; $^{**}P < 0.01$ and $^{***}P < 0.001$ versus no NPs. ψ, pseudouridine. (**g**) Immunostaining for 3xFLAG in lung sections from mice described in **f**. Scale bar, 50 µm. Arrows indicate 3xFLAG expression.

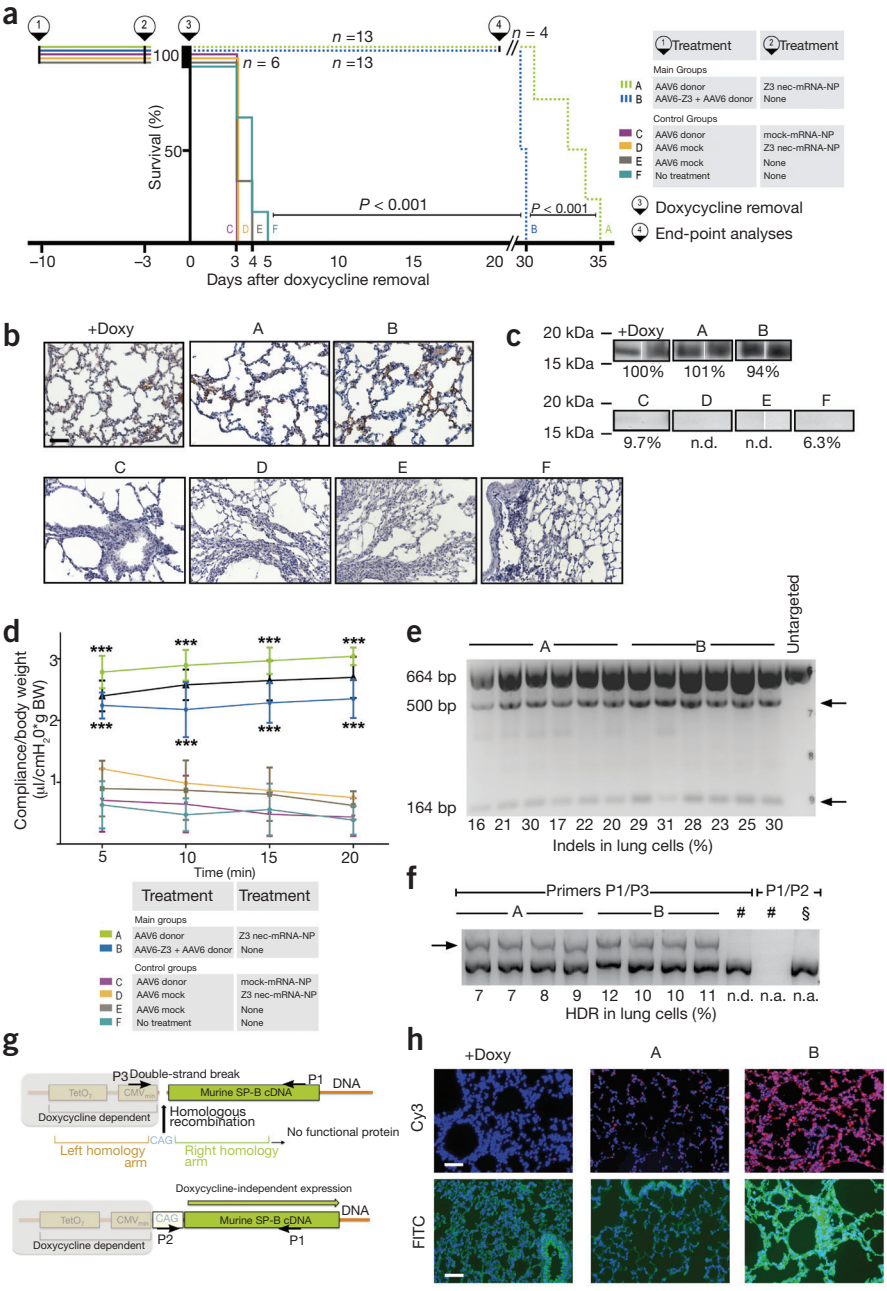

Figure 2 Rescue of SP-B deficient mice by *in vivo* gene manipulation. (**a**) Treatment scheme and Kaplan-Meier survival curves of transgenic SP-B mice treated intratracheally with donor (2.5×10^{11} viral genomes AAV6 donor, AAV6-mock, or none) and nuclease (20 µg Z3 nec-mRNA-NP, mock-mRNA-NP, 5×10^{10} v.g. Z3 AAV or none), then withdrawn from doxycycline. Groups C–F, $n = 6$; groups A and B, $n = 13$, reduced to $n = 4$, 20 d after doxycycline removal. Log-rank tests were performed. (**b**,**c**) Representative SP-B expression (brown) in lung tissue (**b**) and anti-SP-B blots on cell-free broncho-alveolar lavage fluid supernatant (10 µg total protein/lane) (**c**) from mice described in **a**. For complete western blots, see **Supplementary Figure 22**. Scale bar, 50 µm. Lavages and tissue were harvested 20 days after doxycycline removal. $n = 6$ mice per group. (**d**) Lung compliance normalized to respective body weight ($n = 3$, for A or B), 20 d after doxycycline removal. Baseline measurement performed for 20 min; values calculated before each hyperinflation. $^{***}P < 0.001$ versus control groups C–F. Black line with filled triangles: positive control mice on doxycycline. Error bars, mean ± s.d. (**e**,**f**) PCR on lung-isolated DNA from groups A and B or untargeted lungs; each lane represents an individual mouse. Samples were taken 20 d after doxycycline removal. (**e**) PCR of the targeted locus followed by T7 assays. Arrows show expected bands. $n = 6$ mice per group. (**f**) PCR using P1/P3 or P1/P2, followed by gel electrophoresis. #, untargeted control; §, DNA pool of groups A and B. Arrow indicates band resulting from HDR. $n = 4$ mice per group. n.d., not detectable; n.a., not applicable. (**g**) Schematic of the transgenic SP-B cassette, CAG integration and primer (P1, P2 and P3) locations for in-out PCRs. (**h**) Representative immunohistochemistry for groups A, B and a doxycycline-control group (+Doxy) using two different anti-3xFLAG antibodies. Scale bar, 50 µm. Tissue was collected 20 d after doxycycline removal. $n = 6$ mice per group.

immediately upstream of the SP-B cDNA to allow doxycycline-independent expression and prolonged life in treated mice.

First, we customized a panel of zinc finger nucleases (ZFNs) and transcription activator–like effector nucleases (TALENs) targeting the transgenic SP-B cassette (**Fig. 1a** and **Supplementary Fig. 2**). We chose TALEN no. 1 (T-1) and ZFN no. 3 (Z3) owing to their high activity and proximity to the desired site of promoter integration (**Fig. 1a**,**b**) and compared delivery by plasmid DNA and mRNA. mRNA delivery resulted in more frequent induction of double-strand breaks (DSBs) (**Fig. 1c** and **Supplementary Fig. 3**; $P < 0.05$) and homology-directed repair (HDR) (**Fig. 1d**, $P < 0.05$). As Z3 mRNA was more efficient than T-1 mRNA in both cases, Z3 was chosen for further experimentation (amino acid sequences found in **Supplementary Fig. 4**). Comparison with a Z3-encoding AAV serotype 6 vector (AAV6) ("Z3 AAV6") shows the relatively transient expression of Z3 mRNA (**Fig. 1e**), limiting the time during which off-target cleavage activity could occur.

To optimize expression in the mouse lung, we administered a panel of 3xFLAG-tagged Z3 mRNAs with various modification schemes[2,5,10], with or without complexation to biocompatible, biodegradable nanoparticles (NPs) made of chitosan-coated poly (lactic-co-glycolic) acid[11,12]. Following intratracheal (i.t.) delivery, NP-complexation significantly ($P < 0.001$) increased mRNA expression levels (**Supplementary Fig. 5**). 3xFLAG protein expression was most robust for the group with mRNA modified with 25% 2-thiouridine ($s2U_{(0.25)}$) and 25% 5-methylcytidine ($m5C_{(0.25)}$) incorporation and complexed with NP (**Figs. 1f**,**g** and **Supplementary Fig. 6**), and no immune activation was observed after i.t. delivery (**Supplementary Fig. 7**). Therefore, subsequent *in vivo* studies used i.t. delivery of this candidate, called "Z3 nec-mRNA-NP."

Next, we designed a complementary donor template to insert a constitutive CAG promoter at the Z3 nec-mRNA-NP cut site, upstream of the transgenic SP-B cDNA (**Fig. 2g** and **Supplementary Sequences**). Successful site-specific HDR would allow mice to survive and produce SP-B in the absence of doxycycline. As the donor template must be delivered in excess to ensure that it is favored over the homologous chromosome during HDR, we used AAV6, a vector known to transduce lung cells with high efficiency[4]. *Ex vivo* delivery of the AAV6 donor with Z3 nec-mRNA-NP resulted in successful HDR in primary fibroblasts (**Supplementary Fig. 8**). The AAV6 donor and Z3 nec-mRNA-NP (or a Z3 AAV–control) were then delivered in a time-shifted manner to the lungs of transgenic SP-B mice and doxycycline was withdrawn (**Fig. 2a**). Notably, mice in these groups lived significantly longer compared with matched control groups (**Fig. 2a**, $P < 0.001$), while maintaining SP-B expression levels similar to those of mice receiving doxycycline, for as long as 20 days after cessation of doxycycline (**Fig. 2b**,**c** and **Supplementary Figs. 9** and **10**). Combining gene correction with AAV6 donor and Z3 nec-mRNA-NP (or Z3 AAV) prevented the decline in lung function (**Fig. 2d** and **Supplementary Fig. 11**), severe hemorrhagic infiltrations and large-scale edema (**Supplementary Figs. 12–14**), and neutrophilia (**Supplementary Fig. 15**), all of which are observed in the lungs of negative controls. A nonsignificant increase of interleukin (IL)-12 was observed in Z3 nec-mRNA-NP versus PBS-treated mice (**Supplementary Fig. 16**); however, no interferon (IFN)-α elevation was detected (data not shown). Biodistribution analyses revealed that AAV persistence was restricted to the lung, with no detectable expression in heart, liver, kidney or spleen (data not shown). DSB and HDR rates (the latter determined by in-out PCR; **Fig. 2g**) were consistent with successful gene manipulation (**Fig. 2e**,**f**), which was also determined by target-site sequencing (**Supplementary Fig. 17**). If achieved in humans, HDR rates of ~9% (**Fig. 2g**) would likely be sufficient to avoid severe disease progression (**Supplementary Discussion**). These results also confirmed that nuclease expression was longer-lived if administered by AAV, making Z3 nec-mRNA-NP a superior and effective delivery vehicle (**Fig. 2h** and **Supplementary Figs. 18–20**).

In summary, we have shown that delivery of Z3 nec-mRNA complexed to chitosan-coated NPs and AAV6 donor DNA results in successful site-specific genome editing *in vivo*. To our knowledge no previous study has demonstrated life-prolonging gene correction in the lung. Our study has certain limitations, including the need for co-transfection of an AAV-DNA donor template in conjunction with nec-mRNA, the short duration of the cure *in vivo*, probably owing to the natural turnover of the transfected lung cell populations, and the use of a transgenic mouse model in which an artificial cassette is targeted rather than a humanized model. Given the transient nature of nec-mRNA action, this therapeutic modality is best suited to the numerous scenarios in which short-term genome editing can effect long-term clinical benefit.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nbt.3241).

AUTHOR CONTRIBUTIONS

M.S.D.K. designed research, and together with A.J.M. and A.D., carried out experiments and analyzed data. B.M. performed the isolated lung experiments, and together with S.B.-H., participated in the interpretation and analysis of the data, with S.B.-H. and B.N. providing administrative and technical support. M.A., E.M., B.L., L.E.M., J.R., D.M.B., P.R., P.S., F.Z., A.S., M.C. and M.B. conceived and performed experiments. M.G., M.S., R.H., D.H. and C.-M.L. provided materials. M.S.D.K. and L.E.M. wrote the manuscript. All authors discussed results and implications, commented on or edited parts of the manuscript.

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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper (doi:10.1038/nbt.3241).

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ONLINE METHODS

Site-specific nucleases. TALENs and ZFNs targeting the transgenic SP-B cassette were assembled using an archive of zinc-finger proteins, as previously described[13]. The full amino acid sequences of the Z3 pair are shown in **Supplementary Figure 3**. The ZFN expression vector was assembled as previously described[14]. The respective plasmid constructs were kindly provided by Sangamo BioSciences (www.sangamo.com).

Targeting vectors. The targeting vector carrying the CAG promoter was assembled from synthetic oligonucleotides (www.lifetechnologies.com) and PCR products, and was verified by sequencing. The full DNA sequence of the donor vector is depicted in **Supplementary Figure 5**. The NheI restriction fragment length polymorphism (RFLP) donor plasmid was constructed by removing the CAG promoter from the targeting vector by NheI digestion, leaving a single NheI restriction site, which was used in the RFLP assays.

Cell culture and transfection. For the T7 and HDR assays 1×10^6 fibroblasts in 6-well plates were transfected as indicated in the respective figure legends using the Neon electroporation system (www.lifetechnologies.com) with 100 µl tips. The electroporation settings were 1,650 V, 20 ms, 1 pulse. A549 cells (human ATII cells, the cell type responsible for SP-B expression in the lungs) and MLE12 cells (murine ATII cells) were maintained at 37 °C under 5% CO_2 and grown in minimal essential medium (www.lifetechnologies.com), supplemented with 10% FCS, 1% penicillin-streptomycin. One day before transfection, 50,000 or 80,000 cells/well/500 µl were plated in 24-well plates. The cells (70–90% confluent) were transfected with 5 µg (T7 assays, fragment analyses and RFLP) or 1 µg Z3 pair nec-mRNA (time-course experiment) using Neon electroporation (www.lifetechnologies.com) with a transfection mix volume of 100 µl according to manufacturer's instructions or transduced with a multiplicity of infection (MOI) of 1×10^6 v.g. of each Z3 AAV6. For transfection experiments demonstrated in **Figure 1d**, we equilibrated the DNA amounts by adding inert (empty vector) DNA to a total of 9 µg each. For transduction, the cells were washed once with PBS and cultured in OptiMEM; 6 h after transduction 10% FCS was supplied. After 24 h the medium was removed, the cells washed once with PBS, and fresh culture medium added. Primary fibroblasts from transgenic SP-B mice were obtained by removing the dorsal skin, followed by separation of epidermis from the dermis using dispase. After further digestion of the dermis using collagenase, the suspension was passaged through a 70-µm strainer. After the wash and centrifugation steps, the cell pellet was resuspended in fibroblast culture medium (DMEM/Ham's F-12 medium with L-glutamine, 10% MSC grade fetal calf serum, 1× MEM nonessential amino acids, 1× sodium pyruvate, 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol). For the time course experiments: 1, 2, 3, 4, 5 and 14 days after transfection the A549 cells were harvested, permeabilized using BD Cytofix/Cytoperm plus (www.bd.com), stained with APC anti-DYKDDDK clone L5 (www.biolegend.com) antibody and analyzed on an LSR-I flow cytometer (http://www.bd.com/); data were analyzed with BD FACSDiva software (http://www.bd.com/).

Generation of nec-mRNA. To generate templates for *in vitro* transcription the 3xFLAG-tagged T-1 and Z3 were cut out of their original vectors and subcloned into a PolyA-120 containing pVAX1 (www.lifetechnologies.com). The plasmids were linearized with XbaI and transcribed *in vitro* using the MEGAscript T7 Transcription kit (www.lifetechnologies.com), incorporating 25% 2-thio-UTP and 25% 5-methyl-CTP or 100% PseudoUTP and 100% 5-methyl-CTP (all from www.trilinkbiotech.com). The anti reverse CAP analog (ARCA)-capped synthesized nec-mRNAs were purified using the MEGAclear kit (www.lifetechnologies.com) and analyzed for size on agarose gels and for purity and concentration on a NanoPhotometer (http://www.implen.com).

T7 nuclease assay. Genomic DNA was extracted from fibroblasts using the DNeasy Blood & Tissue Kit (www.qiagen.com). A 50-µl PCR reaction was set up using 100 ng of gDNA derived from fibroblasts previously transfected with 5 µg T-1 or Z3 pair, 0.5 µM primers (for T-1: fwd, "P3" GTAGGCGTGTACGGTGGGAG; rev, "P1" CAGCAGAGGGTAGGAAGCAGC; for Z3: fwd, TGTACGGTGGGAGGCCTAT; rev, CCTGGCAGGTGATGTGG), and AmpliTaq Gold 360 Mastermix (www.lifetechnologies.com). Another PCR reaction was performed using the same primer sets, but with gDNA from untransfected cells. The PCR products were run on agarose gels to verify size and sufficient amplification, pooled, purified by ethanol precipitation, dissolved in 20 µl water and the DNA concentration was measured on a NanoPhotometer. 2 µl NEBuffer 2 (www.neb.com), 2 µg purified PCR product and water were brought to a total volume of 19 µl. The DNA was hybridized in a thermocycler according to the following protocol: 95 °C for 5 min, 95–85 °C at –2 °C/s, 85–25 °C at –0.1 °C/s, hold at 4 °C. 1 µl (10 U) of T7E1 (www.neb.com, M0302L) was added and incubated at 37 °C for 15 min. The reaction was stopped by adding 2 µl of 0.25 M EDTA. The reaction was again purified by ethanol precipitation and dissolved in 15 µl water. The nuclease-specific cleavage products were determined on agarose gels. The band intensities were quantified using ImageJ (http://rsb.info.nih.gov/ij/).

For measuring off-target effects, A549 cells were transfected 5 µg mRNA or transduced with 1×10^5 v.g. AAV6-Z3. PCR and T7 was performed as described above (primers: off-target 1: fwd, GCAAGTTTGGCGTCGCTCCA; rev, AGAGGAAGGCGCGGCAGG; off-target 2: fwd, TTCTTGCTCCAGTGACTCTCTTA; rev, AGCCTAGTAAAGACAACACTAGTG; off-target 3: fwd, CAACGTGACCTGCGAGCG; rev, GTGCACGCTCCACTTCTCG; off-target 4: fwd, CTGGAGATGCATCCTTGTCTGT; rev, GAGGGTGAAGACTTTTGGAGCT; off-target 5: fwd, CAGCACCAGATGTTCCCTGTTA; rev, TGGAAAGCAATAGTTCTAGGATGA; off-target 6: fwd, GAGGCTGTGTCACTAGCAGGA; rev, CAAAGTGGTACCTTGGCAAGAG; off-target 7: fwd, AGAAAGCCAGCTGAGTACCA; rev, TGTTGGCTTGTTTGGACTCATG; off-target 8: fwd, TGACTACAATCATGCTTCTTGGTT; rev, TGTAGGCCTTCAGTGATCTAGG; off-target 9: fwd, AAGGACTTCATCTTTGCTGGAT; rev, GAATCAACAGCCTGGCAGC; off-target 10: fwd, ACATTTTCTGGAGTGTAGTGTG; rev, GCTCTTTCGGTAACACAGTTCTT).

HDR/RFLP assay. Genomic DNA was extracted from fibroblasts or lung tissue using the DNeasy Blood & Tissue Kit (www.qiagen.com). T-1 or Z3 target loci were amplified by PCR (40 cycles, 58 °C annealing and 30 s elongation at 72 °C; 5 min at 72 °C to assure completion of amplicons) using 0.5 µM of primers T-1fwd (GTAGGCGTGTACGGTGGGAG) and T-1rev (CAGCAGAGGGTAGGAAGCAGC), as well as P1 and P3 (see above) with AmpliTaq Gold 360 Mastermix. In addition, in-out PCR reactions were performed using primers P1 and P2 (AGGCACTGGGCAGGTAAGTA) (see **Supplementary Fig. 20**).

Flow cytometry. Harvested lungs were digested at 37 °C for 1 h on a rotating shaker in 1 mg/ml collagenase type I (www.lifetechnologies.com), 1% (500 U) DNase (www.epibio.com) solution. Digested lung was passed through a 40-µm nylon cell strainer and erythrocytes were lysed using ACK Lysing Buffer (www.lifetechnologies.com). PE anti-CD45 clone 30-F11, PE anti-CD31 clone C13.3, APC anti-mouse Ly-6A (Sca-1) clone D7 (www.biolegend.com), FITC anti–FLAG M2 and anti-clara cell secretory protein (www.sigmaaldrich.com) were used to stain lung cells. After staining for extracellular markers, cells were fixed and permeabilized using BD Cytofix/Cytoperm plus (www.bd.com), then stained with intracellular antibodies. Flow cytometer analyses were performed on a LSR-I flow cytometer (www.bd.com) and data were analyzed with BD FACSDiva software (www.bd.com).

ATII and Clara cells sorting was performed with a FACSAria (www.bd.com).

Nanoparticles. Chitosan (83% deacetylated (Protasan UP CL 113, www.novamatrix.biz)) coated PLGA (poly-D,L-lactide-co-glycolide 75:25 (Resomer RG 752H, www.evonik.de) nanoparticles (short: NPs) were prepared by using emulsion-diffusion-evaporation[15] with minor changes. In brief, 100 mg PLGA was dissolved in ethyl acetate and added dropwise to an aqueous 2.5% PVA solution (polyvinyl alcohol, Mowiol 4-88, www.kuraray.eu) containing 15 mg Chitosan. This emulsion was stirred (1.5 h at room temperature) and followed by homogenization at 17,000 r.p.m. for 10 min using a Polytron PT 2500E (www.kinematica.ch). These positively charged NPs were sterile filtered and characterized by Malvern ZetasizerNano ZSP (hydrodynamic diameter: 157.3 ± 0.87 nm, PDI 0.11, zeta potential +30.8 ± 0.115 mV). After particle formation they were loaded with mRNA by mixing (weight ratio, 25:1).

AAV vector production. AAV serotype 6 vectors from the Z3 pair and the donor sequence were produced and purchased from Virovek (www.virovek.com).

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Animal experiments. 6- to 8-week-old BALB/c mice (www.criver.com) and transgenic SP-B mice[6] (SP-C rtTA/(teto)$_7$ SP-B/SP-B$^{-/-}$) were maintained under specific pathogen-free conditions and were kept with a 12 h/12 h light/dark cycle. All animals were provided with food and water *ad libitum*, and were acclimatized for at least 7 d before the start of the respective experiment. Transgenic SP-B mice were fed with doxycycline-containing food until cessation (day 0 of the control and main groups). All animal procedures were approved and controlled by the local ethics committee and carried out according to the German law of protection of animal life.

Intratracheal injection. BALB/c or transgenic SP-B mice were anesthetized intraperitoneally with a mixture of medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl (50 μg/kg), and suspended on a mouse intubation platform (www.penncentury.com, Model MIP) at a 45° angle by the upper teeth. A small animal laryngoscope (www.penncentury.com) was used to provide optimal illumination of the trachea. A Microsprayer Aerosolizer - Model IA-1C connected to a FMJ-250 high pressure syringe (both from www.penncentury.com) was endotracheally inserted and either PBS, 20 μg Z3 nec-mRNA, naked or complexed with NPs, or AAV6 (www.virovek.com) was applied in a volume of 100 μl. The Microsprayer tip was withdrawn after 10 s, antidote was injected subcutaneously (atipamezol (50 μg/kg), flumazenil (10 μg/kg) and naloxon (24 μg/kg)), and the mouse was taken off the support after 2 min.

Airway compliance. Compliance was determined by using an *ex vivo* model of the isolated perfused lung as described previously (IPL, Harvard Apparatus)[4,16]. To lower the variability, all mice were treated, and subsequently lungs were isolated within a defined time period. In short, *in situ* mouse lungs were placed in a thorax chamber and mice were ventilated by a tracheal cannula. Ventilation rate was set to 90 breaths per minute with negative pressure ventilation between –2.8 cm H_2O and 8.5 cm H_2O. To prevent atelectasis a hyperinflation was triggered every 5 min (–25 cm H_2O). Perfusion of lungs was done with a 4% hydroxyethyl starch containing perfusion buffer through the pulmonary artery (flow 1 ml/min). Lung function parameters were recorded automatically and compliance calculated by HSE-HA Pulmodyn W Software (Harvard Apparatus). For graphical and statistical analysis, the mean compliance values were calculated from the last ten time stamps (40 s) of each 5-min period (between two hyperinflations). Two mice from group D were too sick to measure and the lung from one mouse from group F was damaged before analysis.

Airway resistance. Airway resistance in response to methacholine (MCh, acetyl-β-methylcholine chloride; Sigma-Aldrich) was again determined using the *ex vivo* model of the isolated perfused lung (IPL, www.harvardapparatus.com)[4,16]. In brief, after a 20-min baseline measurement, lungs were perfused with increasing concentrations of MCh (0.1 μM, 1 μM, 10 μM and 100 μM) for 10 min each, separated by a 10-min washout period with perfusion buffer. Lung function parameters were recorded automatically and airway resistance was recorded by HSE-HA Pulmodyn W Software (www.harvardapparatus.com). For graphical and statistical analysis, the mean resistance values were calculated from the last ten time stamps (40 s) of each 10-min MCh exposure.

Histopathology. Mouse lungs were fixed in 4.5% Histofix (www.carlroth.com) at 4 °C overnight. Fixed lungs were embedded in paraffin, and slices were stained with either H&E or Surfactant Protein-B DAB ((mouse monoclonal anti-SP-B antibody (www.abcam.com, ab3282), Zytochem Plus HRP One-Step Polymer anti-mouse/rabbit/rat (www.zytomed-systems.com, ZUC53-006) and DAB substrate kit for peroxidase (www.vectorlabs.com, SK-4100)). 3xFLAG FITC fluorescence staining (monoclonal anti-FLAG M2-FITC antibody (www.sigma-aldrich.com, F4049)) and DAPI counterstaining (www.applichem.com, A1001) was examined using a Zeiss Axio Imager.

For 3xFLAG Cy3 fluorescence staining, rabbit polyclonal to DDDDK tag antibody (www.abcam.com, ab21536) was used as primary antibody and goat anti-rabbit Cy3 antibody (www.jacksonimmuno.com, 111-165-144) was used as secondary antibody together with DAPI (www.applichem.com, A1001).

Western blot analysis. Protein from broncho-alveolar lavage fluid was separated on NuPAGE 10% Bis-Tris Plus gels and a NuPAGE Mini Gel Tank (all from www.lifetechnologies.com), and immunoblotting was performed by standard procedures according to manufacturer's instructions using the XCell II Mini-Cell and blot modules (www.lifetechnologies.com). After blocking for 2 h at room temperature, primary antibody against SP-B (kindly provided by M.G.) or ANTI-FLAG M2 (www.sigmaaldrich.com) was incubated overnight, horseradish peroxidase–conjugated secondary antibodies (anti-rabbit from www.dianova.com) were incubated for 1 h. Blots were processed by using ECL Prime Western Blot Detection Reagents (www.gelifesciences.com). Semiquantitative analysis was performed with the Quantity One software (www.bio-rad.de).

Target-site sequencing. Genomic DNA from primary fibroblasts (*in vitro* transfected/transduced) or sorted ATII cells (after *in vivo* transfection/transduction) was isolated using the NucleoSpin Tissue Kit (www.mn-net.com) according to the manufacturer's protocol. Amplicons were derived from PCR with Primers P1 and P2 (see sequences above) using the following conditions: AmpliTaq Gold 360 master mix (www.lifetechnologies.com) at 95 °C for 10 min, 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, with total 35 cycles and a final extension step at 72 °C for 7 min. The amplicons were cloned into the pCR-TOPO vector (www.lifetechnologies.com) and sequenced using the primers M13forward (`GTAAAACGACGGCCAGTG`) and M13reverse (`CAGGAAACAGCTATGACCATG`). The alignments have been performed with Geneious R6 (www.biomatters.com) using the "multiple align" function, choosing a cost matrix of 65% similarity (5.0/–4.0), a gap open penalty of 12 and a gap extension penalty of 3.

Real-time RT-PCR. The lung cell separations were washed vigorously three times with PBS to avoid carrying over RNA not taken up by lung cells (the third supernatant was later tested for RNA contamination using the qPCR procedure described below). RNA was then isolated with the RNeasy purification kit (www.qiagen.com). Reverse transcription of 50 ng RNA was carried out using iScript cDNA synthesis kit (www.bio-rad.com). Detection of Z3 cDNA was performed by SYBR-Green based quantitative Real-time PCR in 20-μl reactions on a ViiA7 (www.lifetechnologies.com). Reactions were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 2 min at 50 °C (annealing and extension), followed by standard melting curve analysis. The following primer pairs were used: Z3 left fwd `TGTACGGCTACAGGGGAA`, Z3 left rev `GCCGATAGGCAGATTGTA`; optimal determined house-keeping gene beta-actin: fwd `TAGGCACCAGGGTGATG`, rev `GCCATGTTCAATGGGGTACT`.

Statistics. Differences in mRNA expression between groups were analyzed by pair-wise fixed reallocation randomization tests with REST 2009 software[17]. All other analyses were performed using the Wilcoxon-Mann-Whitney test with SPSS 21 (www.ibm.com). Data are presented as mean ± s.e.m. or as the median ± IQR (interquartile ranges) and $P < 0.05$ (two-tailed) was considered statistically significant. For survival studies log-rank tests were performed. Statistics for lung compliance was performed using two-way ANOVA and Bonferroni-post tests with GraphPad Prism 5.0 software. Lung function data are presented as mean ± s.d. and $P < 0.05$ (two-tailed) was considered statistically significant. No randomization was used for animal experiments. In all cases but at administration of AAV6/mRNA intratracheally, the investigators were blinded when assessing outcomes.

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