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

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To the Editor:

Nuclease-mediated genome editing 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 non-integrating 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 it to deliver site-specific nucleases, demonstrating the value of nuclease-encoding chemically modified (nec) mRNA as a tool for *in vivo* genome editing. We chose 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 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 immediately upstream of the SP-B cDNA to allow doxycycline-independent expression and prolonged life in treated mice.

First, we customized a panel of ZFNs and TALENs targeting the transgenic SP-B cassette (**Fig. 1a** and **Supplementary Fig. 2**). We chose TALEN #1 (T1) and ZFN #3 (Z3) owing to their high activity and proximity to the desired site of promoter integration (**Figs. 1a,b;** amino acid sequences in **Supplementary Fig. 4**) and compared delivery by plasmid

DNA and mRNA. mRNA delivery resulted in higher levels of double-strand break (DSB)-induction (**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 T1 mRNA in both cases, Z3 was chosen for further experimentation. Comparison with a Z3-encoding AAV serotype 6 vector (AAV6) ("Z3 AAV") shows the relatively transient expression of Z3 mRNA (**Fig. 1e**), limiting the time during which off-target cleavage activity could occur.

To optimize Z3 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 (chit-PLGA)[11, 12]. Following intratracheal (i.t.) delivery, NP-complexation significantly increased mRNA expression levels (**Supplementary Fig. 5**). 3xFLAG protein expression was most robust for the $s2U_{0.25}m5C_{0.25}$-modified, NP-complexed group (**Figs. 1f,g** and **Supplementary Fig. 6**), and no immune activation was observed following 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 **Section B** in the **Supplementary Materials**). Successful site-specific HDR would allow mice to survive and produce SP-B in the absence of doxycycline. As it is critical to deliver the donor template in excess to ensure it is favored over the homologous chromosome during HDR, we used a vector known to transduce lung cells with high efficiency[4], AAV6 (integration-deficient lentiviruses[13] will be tested in future studies). *Ex vivo* delivery of the AAV6-donor with Z3 nec-mRNA-NP resulted in successful HDR in primary fibroblasts (**Supplementary Fig. 8**). AAV6-donor and Z3 nec-mRNA-NP (or a Z3 AAV positive control) were then delivered to the lung of transgenic SP-B mice, followed by cessation of doxycycline (**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 mice receiving doxycycline, as far as 20d after cessation of doxycycline (**Figs. 2b,c** and **Supplementary Figs. 9** and **10**). Phenotypically, 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**) observed in the lungs of negative controls. A non-significant increase of IL-12 was observed in nec-mRNA-NP- versus PBS-treated mice (**Supplementary Fig. 16**); however, no 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, see **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 (see **Supplementary Discussion**). Our results also confirmed that nuclease expression was longer-lived if administered via AAV, making nec-mRNA-NP a superior delivery vehicle (**Fig. 2h** and **Supplementary Figs. 18** and **19**).

Our approach 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 targeting an artificial cassette as opposed to a humanized model. However, we envision the use of optimized nec-mRNA systems with any nuclease, including CRISPR(-Cas9), for targeted gene knock-outs and gene correction in the treatment of SP-B deficiency and a variety of other diseases, such as cancer.

In summary, we have shown that co-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.

Figure 1. nec-mRNA cleaves the SP-B cassette, induces HDR *in vitro*, and is expressed in lung cells *in vivo*. (**a**) TALEN and ZFN candidates relative to the transgenic SP-B cassette. Transgenic SP-B mice-derived fibroblasts were used for (**b-d**); n.d., not detectable. (**b**) T7 assays to determine the frequency of TALEN- and ZFN-induced indels in genomic DNA harvested 3d post-transfection (5μg/ 80,000-100,000 cells). (**c**) T1- and Z3-induced indels following delivery as either mRNA or pDNA (0.5 or 5μg). (**d**) Percent HDR 3d following co-transfection of 5μg T1 or Z3 mRNA (or pDNA) with 0-4μg donor plasmid. Arrows denote *Nhe*I-sensitive cleavage products resulting from HDR. (**e**) Time-course showing kinetics and stability of 3xFLAG-tagged Z3 mRNA versus Z3 AAV in A549 cells (n=3). (**f**) Anti-3xFLAG flow cytometry shows protein expression in total lung cells and ATII cells.

Boxes, medians ± IQRs; whiskers, minimum and maximum; *, $P < 0.05$ versus unmodified; ** and ***, $P < 0.01$ and $P < 0.001$ versus no NPs. (**g**) Immunostaining for 3xFLAG in lung sections from mice described in **f**. Scale bar, 50μm. 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 i.t. with donor ($2.5x10^{11}$ v.g. AAV6-donor, AAV6-mock, or none) and nuclease (20μg Z3 nec-mRNA-NP, mock-mRNA-NP, $5x10^{10}$ 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 20d post-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 BALF supernatant (10μg total protein/lane) (**c**) from mice described in **a**. Scale bar, 50μm. 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), 20d after doxycycline removal. Baseline measurement performed for 20min; values

calculated prior to each hyperinflation. ***, $P < 0.001$ versus control groups C-F;.Black line with filled triangles: positive control mice on doxycycline. (**e,f**) PCR on lung-isolated DNA from groups A and B or untargeted lungs; each lane represents an individual mouse. Samples were taken 20d 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. (**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, 50µm. Tissue was collected 20d after doxycycline removal. n=6 mice per group.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

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., G.D., 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

M.S.D.K. and L.E.M. are listed as co-inventors on a patent application related to this work. M.S.D.K. is an inventor on a patent licensed to the biopharmaceutical company, Ethris GmbH.

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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[15]. The full amino acid sequences of the Z3 pair are shown in Supplementary Fig. 3. The ZFN expression vector was assembled as previously described[16]. 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 Fig. 5. The *Nhe*I RFLP donor plasmid was constructed by removing the CAG promoter from the targeting vector by *Nhe*I digestion, leaving a single *Nhe*I restriction site, which was used in the RFLP assays.

Cell culture and transfection. For the T7 and HDR assays $1x10^6$ fibroblasts in 6-well plates were transfected as indicated in the respective figure legends using the Neon electroporation system (www.lifetechnologies.com) with 100µl tips. The electroporation settings were 1,650V, 20ms, 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/500µl were plated in 24-well plates. The cells (70-90% confluent) were transfected with 5µg (T7 assays, fragment analyses and RFLP) or 1µg Z3 pair nec-mRNA (time-course experiment) using Neon electroporation (www.lifetechnologies.com) with a transfection mix volume of 100µl according to manufacturer's instructions or transduced with MOI of $1x10^6$ v.g. of each Z3 AAV6. For transfection experiments demonstrated in Fig. 1d, we equilibrated the DNA amounts by adding inert (empty vector) DNA to a total of 9µg each. For transduction, the cells were washed once with PBS and cultured in OptiMEM; 6 h after transduction 10% FCS was supplied. After 24h the medium was removed, the cells washed once with PBS and fresh culture medium was 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 70µm strainer. After 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, 1x MEM non-essential amino acids, 1x sodium pyruvate, 1% penicillin/streptomycin, 0.1mM 2-mercaptoethanol). For the time course experiments: after 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 analysed on an LSR-I flow cytometer (www.bd.com) and data were analysed with BD FACSDiva software (www.bd.com).

Generation of (nec-)mRNA. To generate templates for *in vitro* transcription the 3xFLAG-tagged T1 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 *Xba*I 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.trilink.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 (www.implen.com).

T7 nuclease assay. Genomic DNA was extracted from fibroblasts using the DNeasy Blood & Tissue Kit (www.qiagen.com). A 50µl PCR reaction was set up using 100ng of gDNA

derived from fibroblasts previously transfected with 5µg T1 or Z3 pair, 0.5 µM primers (for T1: 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 20µl water and the DNA concentration was measured on a NanoPhotometer. 2µl NEBuffer 2 (www.neb.com), 2µg purified PCR product and water were brought to a total volume of 19µl. The DNA was hybridized in a thermocycler according to the following protocol: 95°C for 5 min, 95-85°C at -2°C/sec, 85-25°C at -0.1°C/sec, hold at 4°C. 1µl (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 $1x10^5$ 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-taget 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). T1 or Z3 target loci were amplified by PCR (40 cycles, 58°C annealing and 30 sec elongation at 72°C; 5 min at 72°C to assure completion of amplicons) using 0.5 µM of primers T1fwd (GTAGGCGTGTACGGTGGGAG) and T1rev (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).

Flow Cytometry. Harvested lungs were digested at 37°C for 1 hour 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 40µm nylon cell strainer and erythrocytes were lysed using ACK Lysing Buffer (www.lifetechnologies). 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 analysed with BD FACSDiva software (www.bd.com).
ATII and Clara cells sorting were 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[17] with minor changes. In brief, 100mg 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 15mg Chitosan. This emulsion was stirred (1.5 h at RT) and followed by homogenization at 17,000 rpm for 10 min using a Polytron PT 2500E (www.kinematica.ch). These positive charged NPs were sterile filtered and characterized by Malvern ZetasizerNano ZSP

(hydrodynamic diameter: 157.3±0.87nm, PDI 0.11, zeta potential +30.8 ± 0.115mV). 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).

Animal experiments. 6-8 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 7d 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 (0.5mg/kg), midazolam (5mg/kg) and fentanyl (50µg/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 PBS, 20µg Z3 (nec-)mRNA naked or complexed with Nanoparticles or AAV6 (www.virovek.com) (was applied in a volume of 100µl. The Microsprayer tip was withdrawn after 10 sec, antidot was injected subcutaneously (atipamezol (50µg/kg), flumazenil (10µg/kg) and naloxon (24µg/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, 18]. 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 via 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 minutes (-25 cm H_2O). Perfusion of lungs was done with a 4% hydroxyethyl starch containing perfusion buffer via the pulmonary artery (flow 1ml/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 10 timestamps (40 sec) of each 5-minute 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 prior to 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, 18]. In brief, after a 20-minutes baseline measurement, lungs were perfused with increasing concentrations of MCh (0.1µM, 1µM,
10µM, and 100µM) for 10 minutes each, separated by a 10-minute 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 10 timestamps (40 sec) of each 10-minute 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.com, ZUC53-006) and DAB substrate kit for peroxidase (www.vectorlabs.com, SK-4100)). 3xFLAG

FITC fluorescence staining ((monoclonal anti-FLAG M2-FITC antidbody (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. Protein from BALF 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 hours at room temperature, primary antibody against SP-B (kindly provided by Prof. Griese, Munich) or ANTI-FLAG M2 (www.sigmaaldrich.com) was incubated overnight, HRP-conjugated secondary antibodies (anti rabbit from www.dianova.com) were incubated for 1h. 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 (sequences see above) using the following conditions: AmpliTaq Gold 360 master mix (www.lifetechnologies.com) at 95 °C for 10 min, 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 60 sec, with in 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.

RealTime 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 sec 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[19]. 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 2way 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 i.t., the investigators were blinded when assessing outcomes.