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Supplemental Information

Highly Efficient Gene Editing of Cystic Fibrosis Patient-Derived Airway Basal Cells Results in Functional CFTR Correction

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Fig. S1

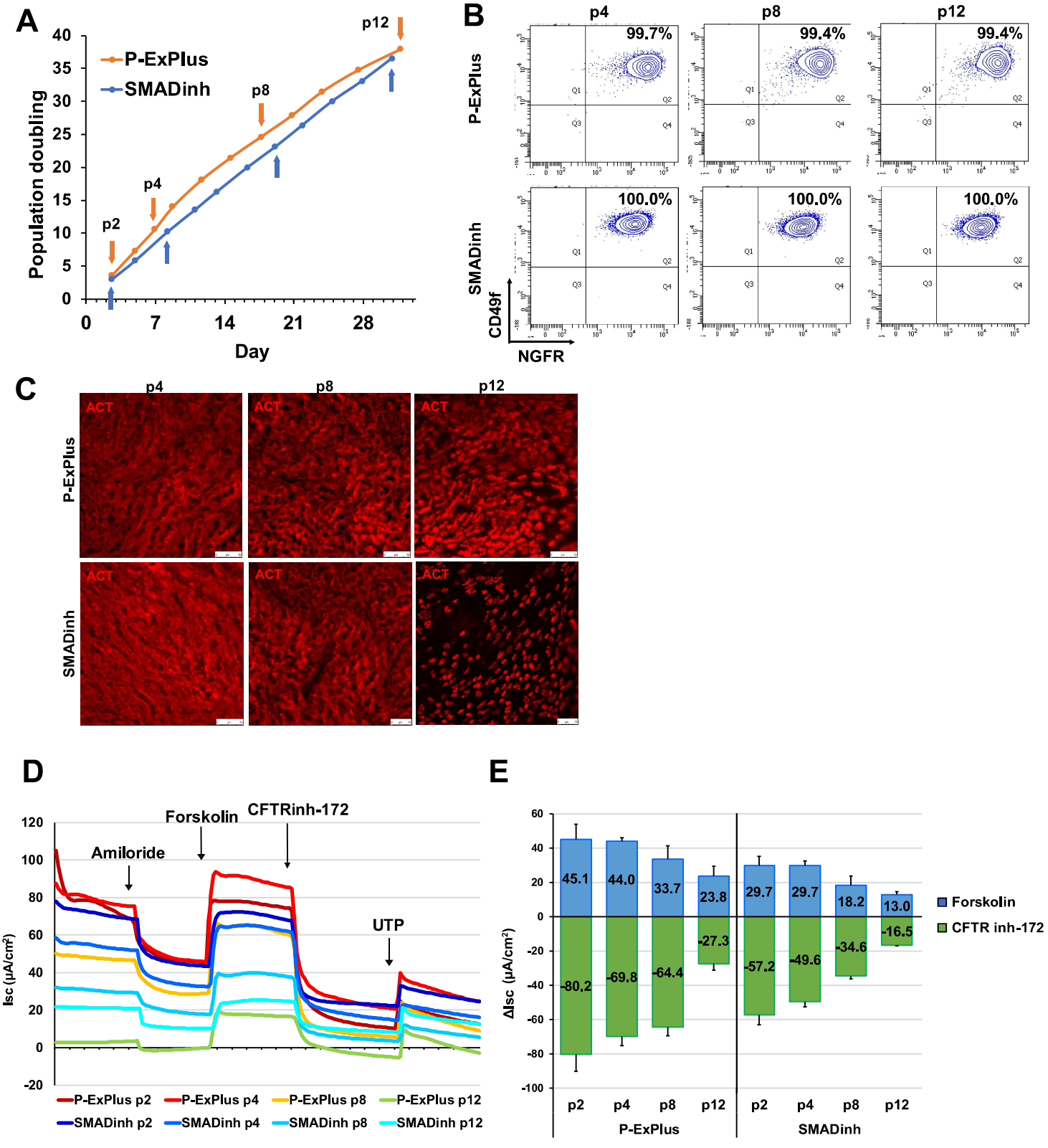

A
Population doubling
Day
P-ExPlus
SMADinh
p2
p4
p8
p12
B
p4
p8
p12
P-ExPlus
SMADinh
99.7%
99.4%
99.4%
100.0%
100.0%
100.0%
CD49f
NGFR
C
p4
p8
p12
P-ExPlus
SMADinh
ACT
D
Forskolin
CFTRinh-172
Amiloride
UTP
Isc (µA/cm²)
P-ExPlus p2
P-ExPlus p4
P-ExPlus p8
P-ExPlus p12
SMADinh p2
SMADinh p4
SMADinh p8
SMADinh p12
E
ΔIsc (µA/cm²)
45.1
44.0
33.7
23.8
29.7
29.7
18.2
13.0
-80.2
-69.8
-64.4
-27.3
-57.2
-49.6
-34.6
-16.5
p2
p4
p8
p12
P-ExPlus
SMADinh
Forskolin
CFTR inh-172

Fig. S2

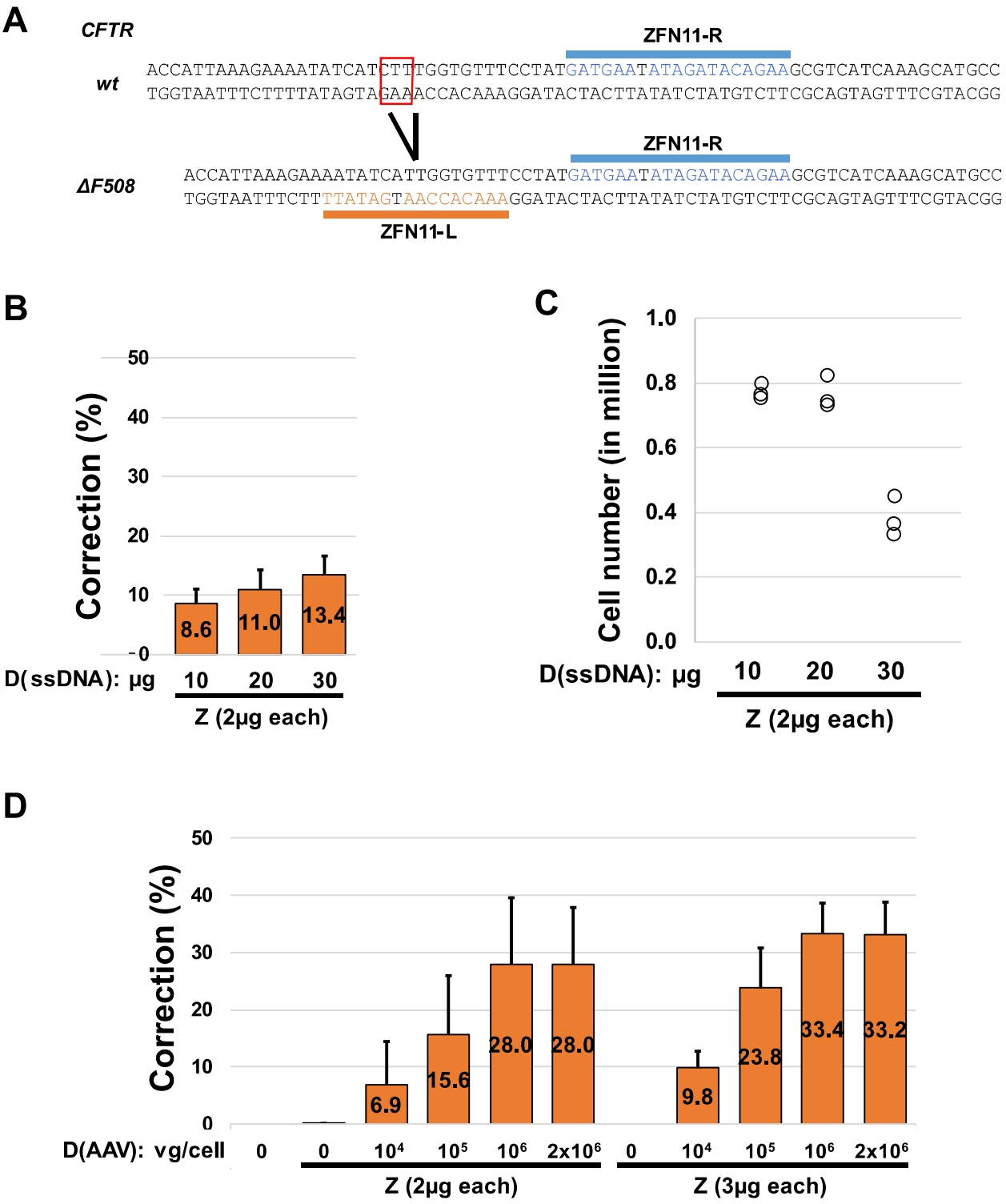
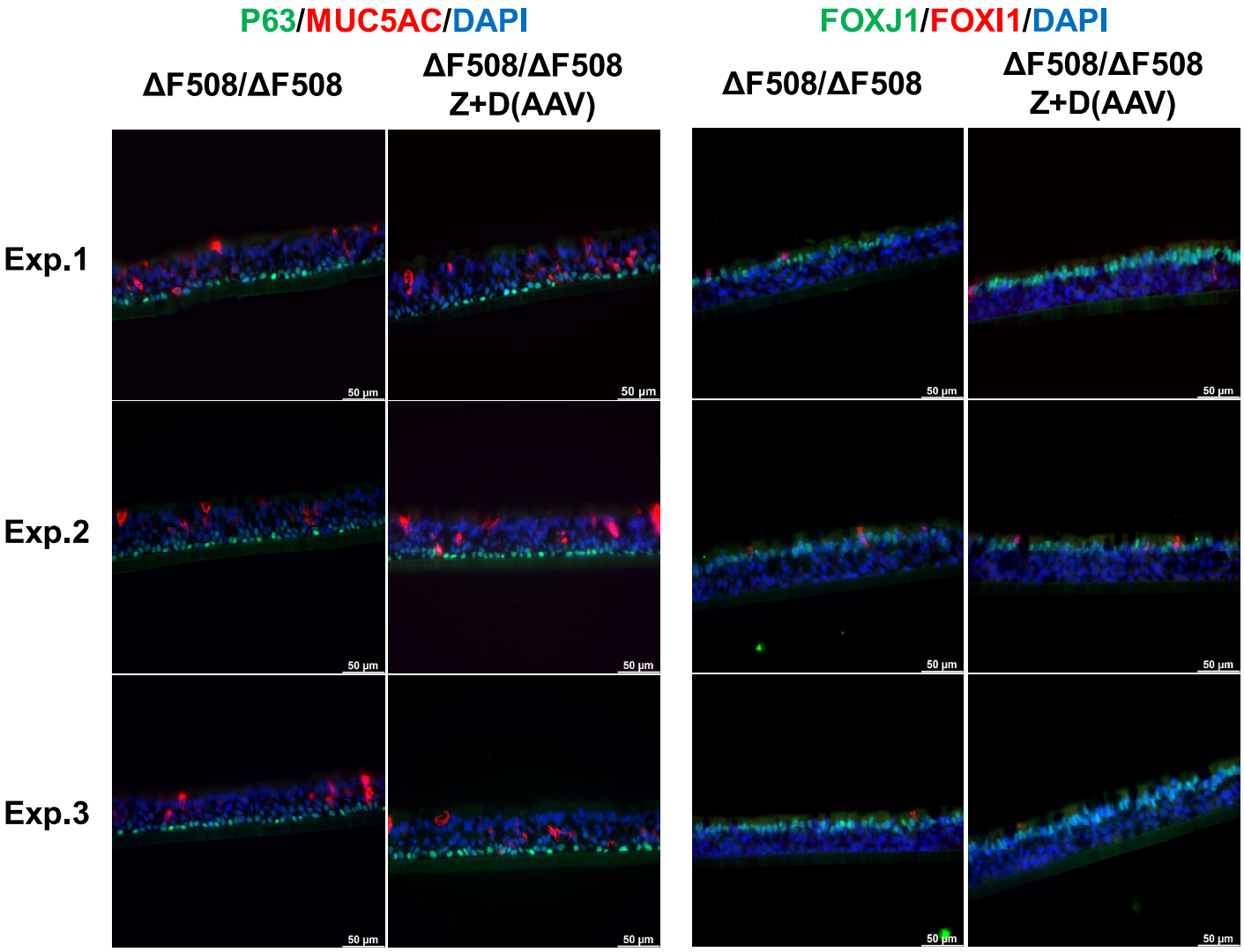

A
CFTR
ZFN11-R
wt
ACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCC
TGGTAATTTCTTTTATAGTAGAAACCACAAAGGATACTACTTATATCTATGTCTTCGCAGTAGTTTCGTACGG
ZFN11-R
ΔF508
ACCATTAAAGAAAATATCATTGGTGTTTCCTATGATGAATATAGATACAGAAGCGTCATCAAAGCATGCC
TGGTAATTTCTTTTATAGTAACCACAAAGGATACTACTTATATCTATGTCTTCGCAGTAGTTTCGTACGG
ZFN11-L
B
Correction (%)
50
40
30
20
10
0
8.6
11.0
13.4
D(ssDNA): μg
10
20
30
Z (2μg each)
C
Cell number (in million)
1.0
0.8
0.6
0.4
0.2
0.0
D(ssDNA): μg
10
20
30
Z (2μg each)
D
Correction (%)
50
40
30
20
10
0
6.9
15.6
28.0
28.0
9.8
23.8
33.4
33.2
D(AAV): vg/cell
0
0
10⁴
10⁵
10⁶
2x10⁶
0
10⁴
10⁵
10⁶
2x10⁶
Z (2μg each)
Z (3μg each)

Fig. S3


P63/MUC5AC/DAPI
FOXJ1/FOXI1/DAPI
ΔF508/ΔF508
ΔF508/ΔF508 Z+D(AAV)
ΔF508/ΔF508
ΔF508/ΔF508 Z+D(AAV)
Exp.1
Exp.2
Exp.3
50 μm

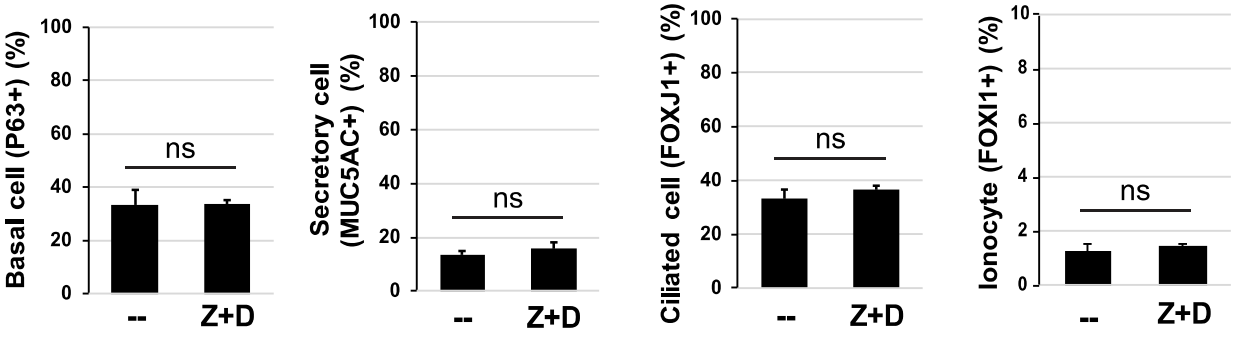

Basal cell (P63+) (%)
Secretory cell (MUC5AC+) (%)
Ciliated cell (FOXJ1+) (%)
Ionocyte (FOXI1+) (%)
ns
--
Z+D

Fig. S4

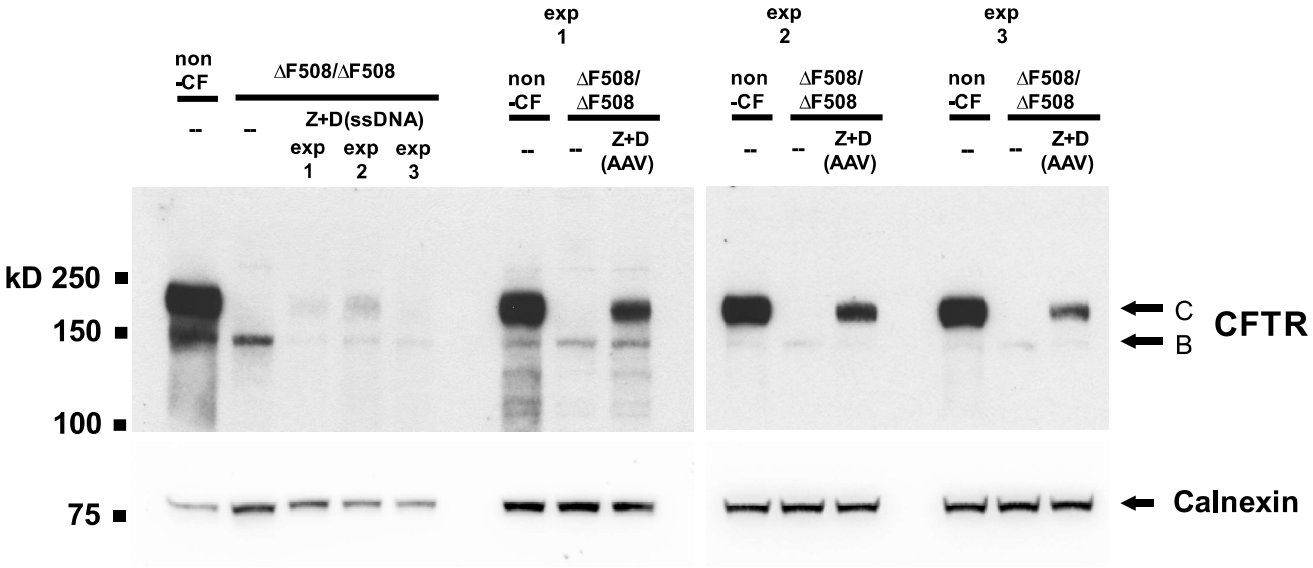

exp 1
exp 2
exp 3
non -CF
ΔF508/ΔF508
Z+D(ssDNA)
exp 1
exp 2
exp 3
non -CF
ΔF508/ ΔF508
Z+D (AAV)
non -CF
ΔF508/ ΔF508
Z+D (AAV)
non -CF
ΔF508/ ΔF508
Z+D (AAV)
kD 250
150
100
75
C
B
CFTR
Calnexin

Fig. S5

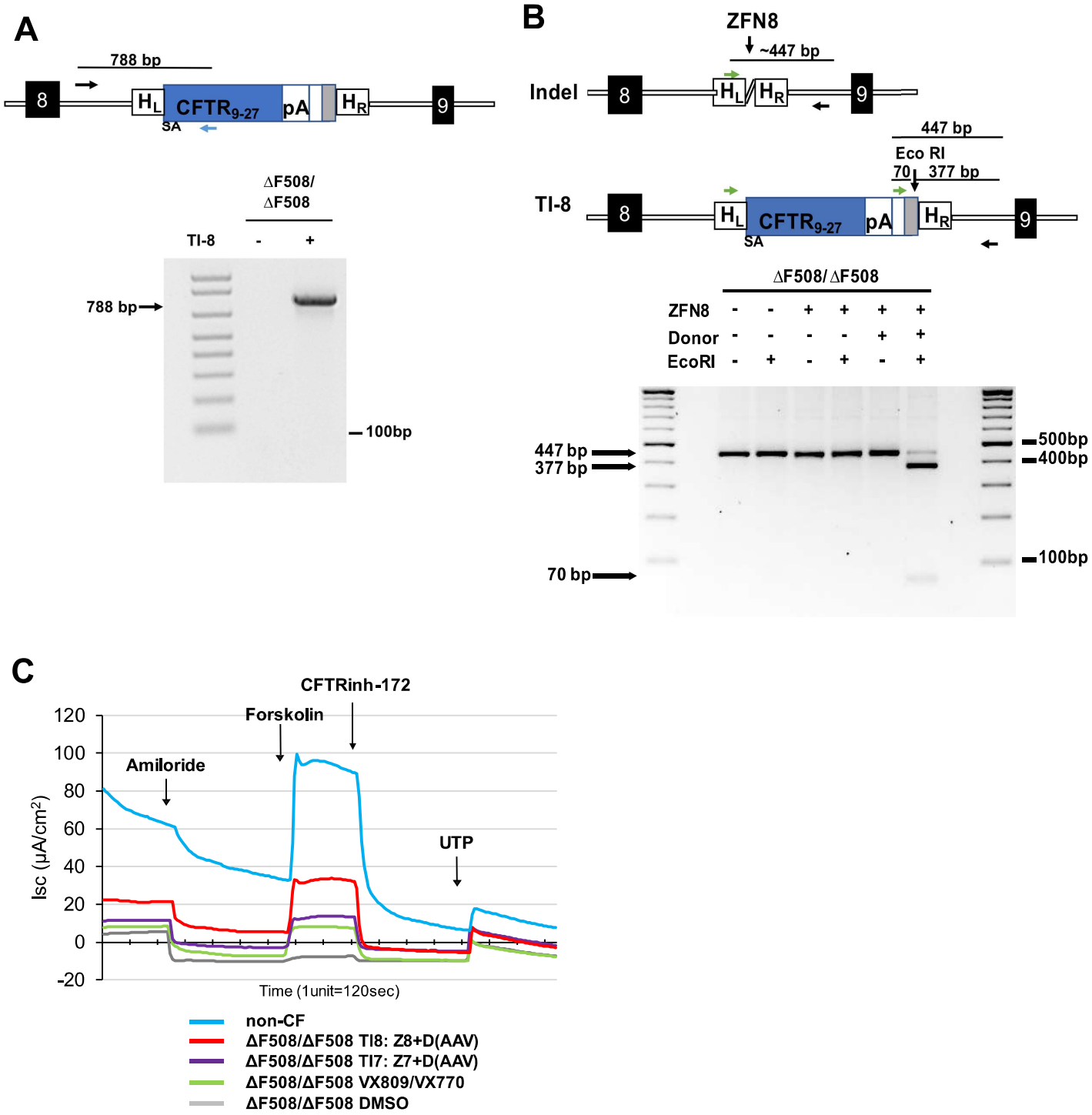
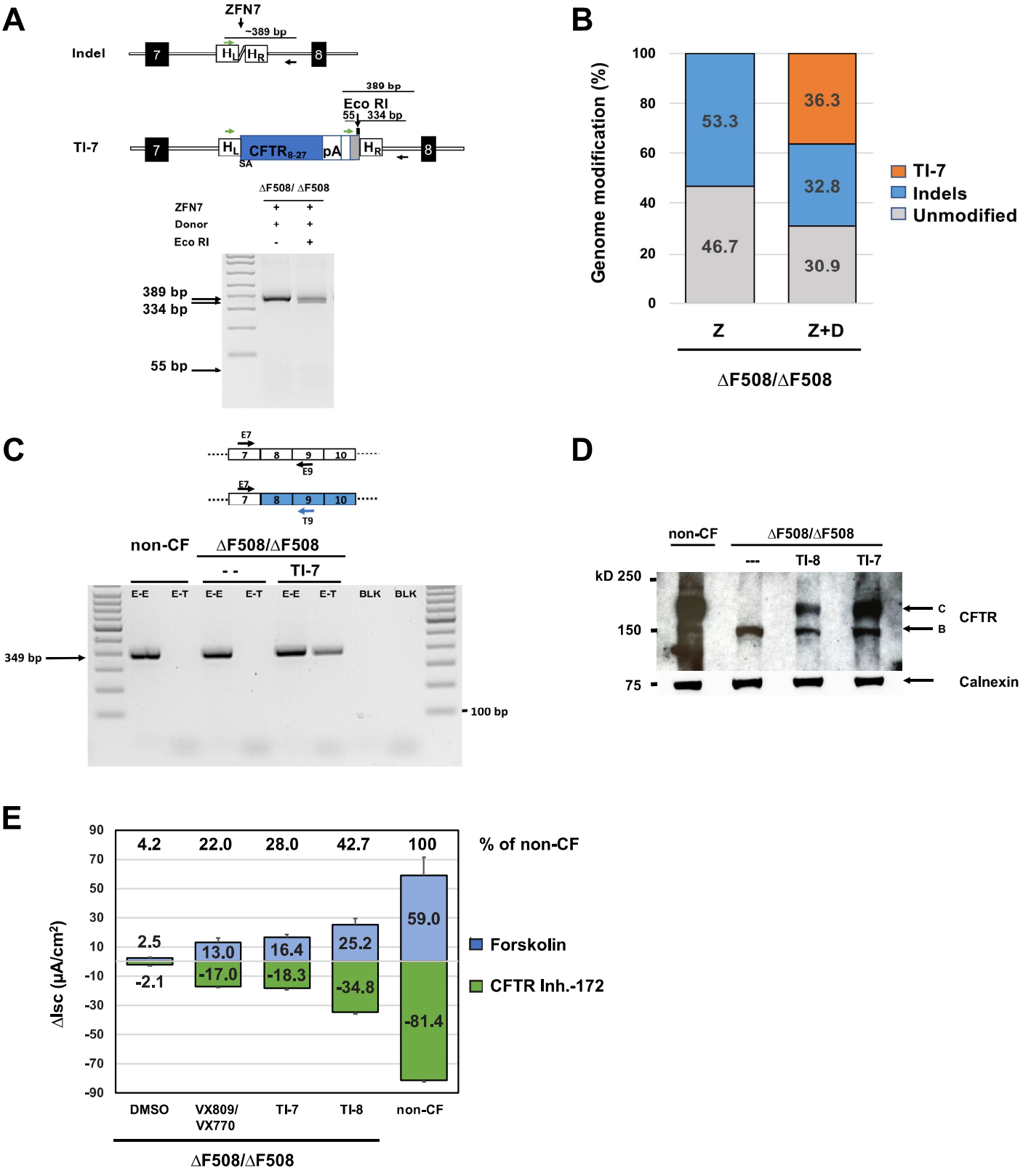
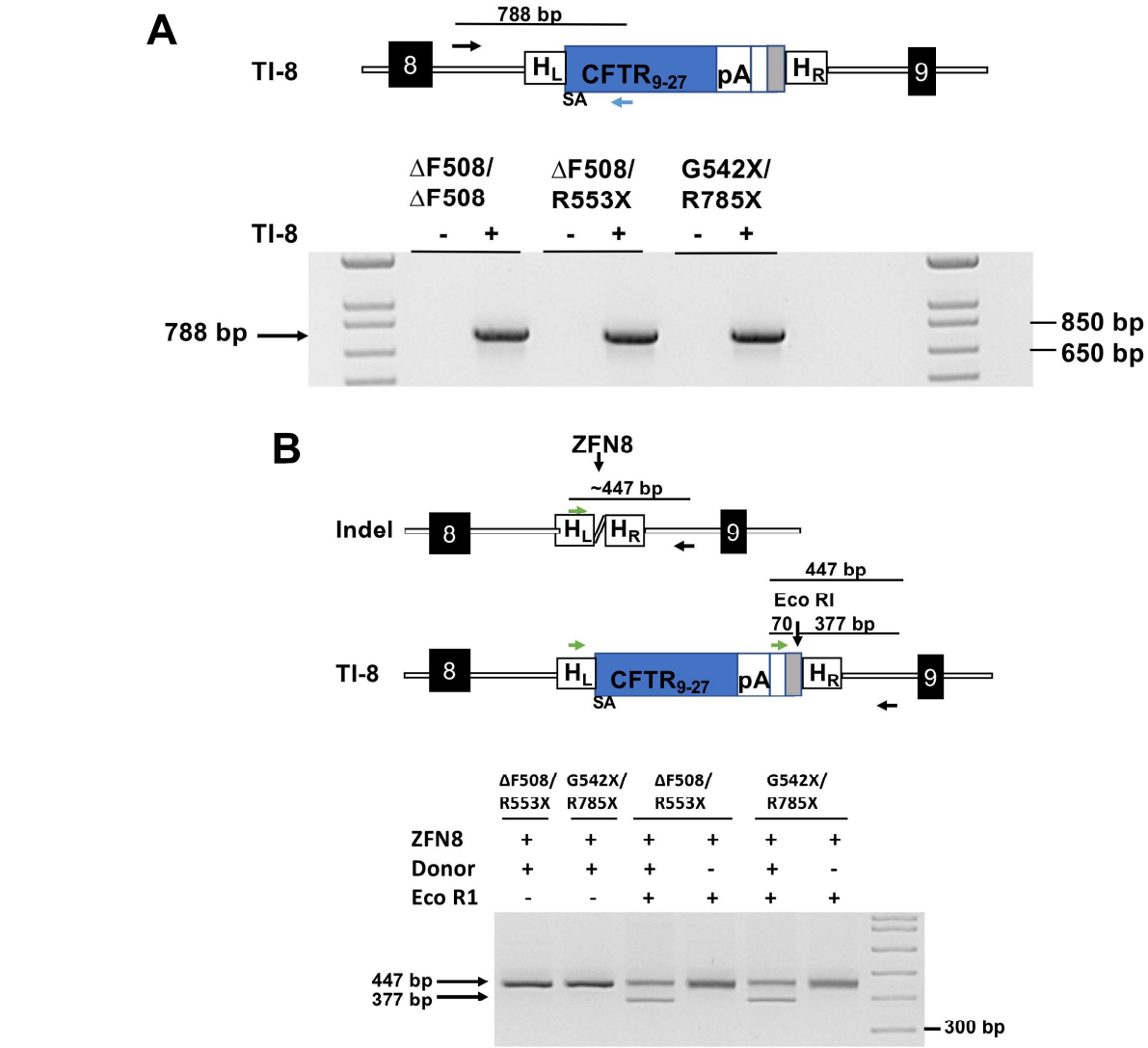

A
788 bp
8
HL
CFTR9-27
pA
HR
9
SA
ΔF508/
ΔF508
TI-8 - +
788 bp
100bp
B
ZFN8
~447 bp
Indel
8
HL
HR
9
447 bp
Eco RI
70 377 bp
TI-8
8
HL
CFTR9-27
pA
HR
9
SA
ΔF508/ΔF508
ZFN8 - - + + + +
Donor - - - - + +
EcoRI - + - + - +
447 bp
377 bp
70 bp
500bp
400bp
100bp
C
CFTRinh-172
Forskolin
Amiloride
UTP
Isc (μA/cm2)
120
100
80
60
40
20
0
-20
Time (1unit=120sec)
non-CF
ΔF508/ΔF508 TI8: Z8+D(AAV)
ΔF508/ΔF508 TI7: Z7+D(AAV)
ΔF508/ΔF508 VX809/VX770
ΔF508/ΔF508 DMSO

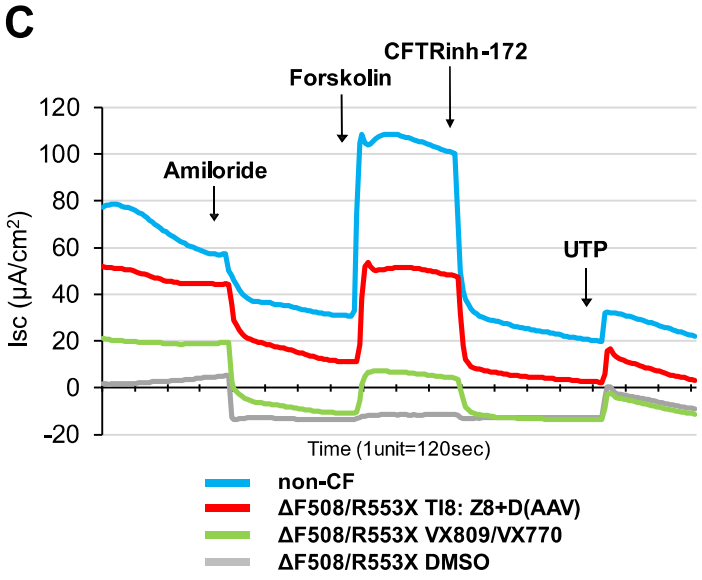
Fig. S6


A
ZFN7
~389 bp
Indel
7
HL
HR
8
389 bp
Eco RI
55
334 bp
TI-7
CFTR8-27
SA
pA
ΔF508/ ΔF508
ZFN7
Donor
Eco RI
389 bp
334 bp
55 bp
B
Genome modification (%)
100
80
60
40
20
0
53.3
46.7
36.3
32.8
30.9
Z
Z+D
ΔF508/ΔF508
TI-7
Indels
Unmodified
C
E7
7
8
9
10
E9
T9
non-CF
ΔF508/ΔF508
- -
TI-7
E-E
E-T
BLK
349 bp
100 bp
D
non-CF
ΔF508/ΔF508

TI-8
TI-7
kD 250
150
75
C
B
CFTR
Calnexin
E
% of non-CF
4.2
22.0
28.0
42.7
100
90
70
50
30
10
-10
-30
-50
-70
-90
ΔIsc (μA/cm²)
2.5
-2.1
13.0
-17.0
16.4
-18.3
25.2
-34.8
59.0
-81.4
Forskolin
CFTR Inh.-172
DMSO
VX809/
VX770
TI-7
TI-8
non-CF
ΔF508/ΔF508

Fig. S7


A
788 bp
TI-8
8
HL
CFTR9-27
pA
HR
9
SA
ΔF508/ ΔF508
ΔF508/ R553X
G542X/ R785X
TI-8
- + - + - +
788 bp
850 bp
650 bp
B
ZFN8
~447 bp
Indel
8
HL/HR
9
447 bp
Eco RI
70 377 bp
TI-8
8
HL
CFTR9-27
pA
HR
9
SA
ΔF508/ R553X
G542X/ R785X
ΔF508/ R553X
G542X/ R785X
ZFN8 + + + + + +
Donor + + + - + -
Eco R1 - - + + + +
447 bp
377 bp
300 bp


C
CFTRinh-172
Forskolin
Amiloride
UTP
Isc (μA/cm²)
120
100
80
60
40
20
0
-20
Time (1unit=120sec)
non-CF
ΔF508/R553X TI8: Z8+D(AAV)
ΔF508/R553X VX809/VX770
ΔF508/R553X DMSO

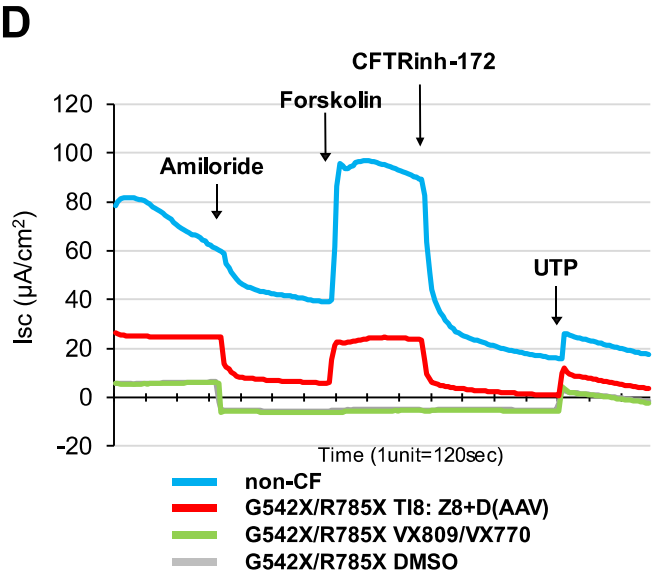

D
CFTRinh-172
Forskolin
Amiloride
UTP
Isc (μA/cm²)
120
100
80
60
40
20
0
-20
Time (1unit=120sec)
non-CF
G542X/R785X TI8: Z8+D(AAV)
G542X/R785X VX809/VX770
G542X/R785X DMSO

Fig. S8

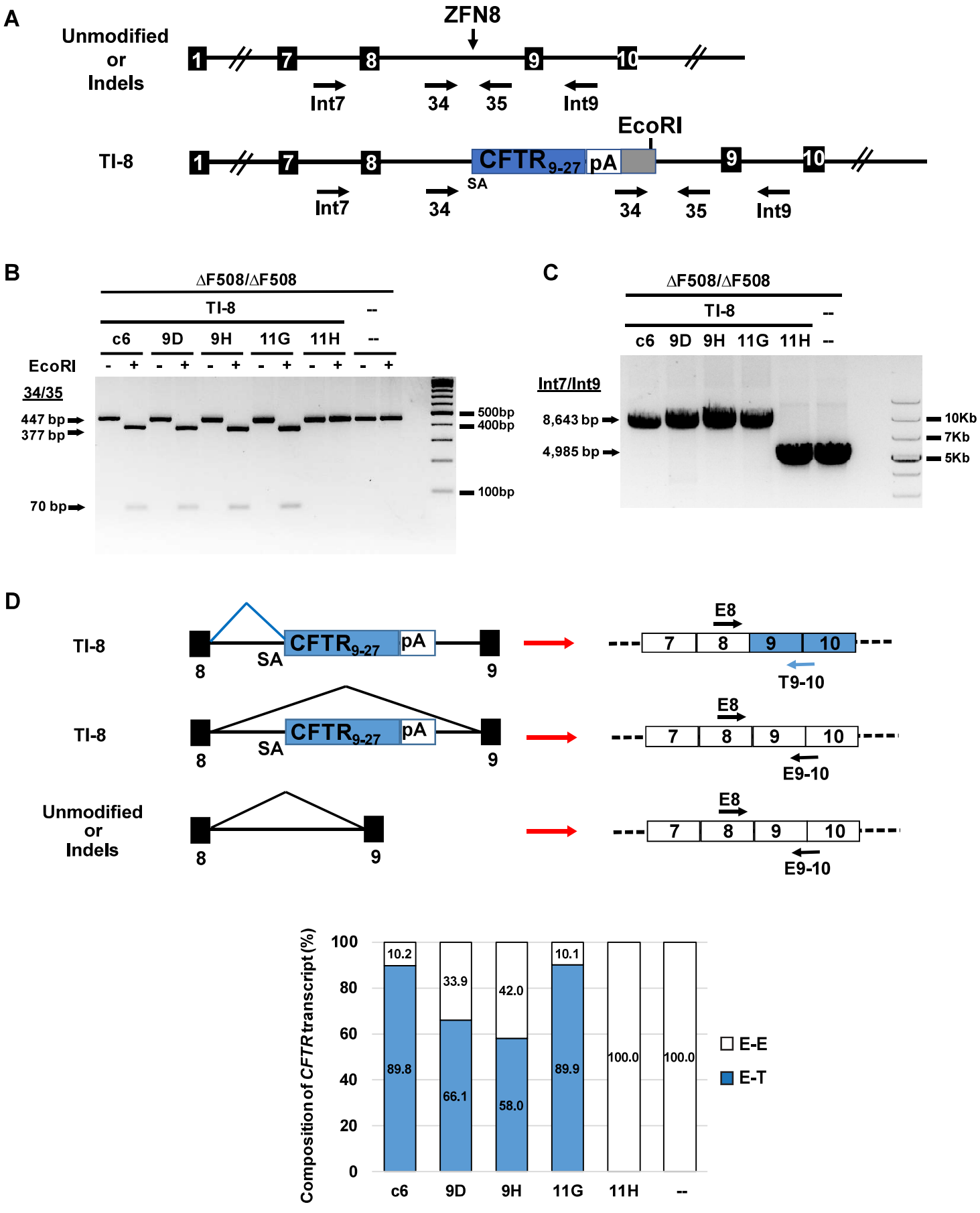

A
Unmodified or Indels
ZFN8
1
7
8
9
10
Int7
34
35
Int9
TI-8
EcoRI
CFTR9-27
pA
SA
34
35
B
ΔF508/ΔF508
TI-8
--
c6
9D
9H
11G
11H
--
EcoRI
- + - + - + - + - + - +
34/35
447 bp
377 bp
70 bp
500bp
400bp
100bp
C
ΔF508/ΔF508
TI-8
--
c6 9D 9H 11G 11H --
Int7/Int9
8,643 bp
4,985 bp
10Kb
7Kb
5Kb
D
TI-8
SA
CFTR9-27
pA
8
9
E8
7
8
9
10
T9-10
TI-8
E9-10
Unmodified or Indels
Composition of CFTR transcript (%)
100
80
60
40
20
0
10.2
89.8
33.9
66.1
42.0
58.0
10.1
89.9
100.0
100.0
c6
9D
9H
11G
11H
--
E-E
E-T

Fig. S9

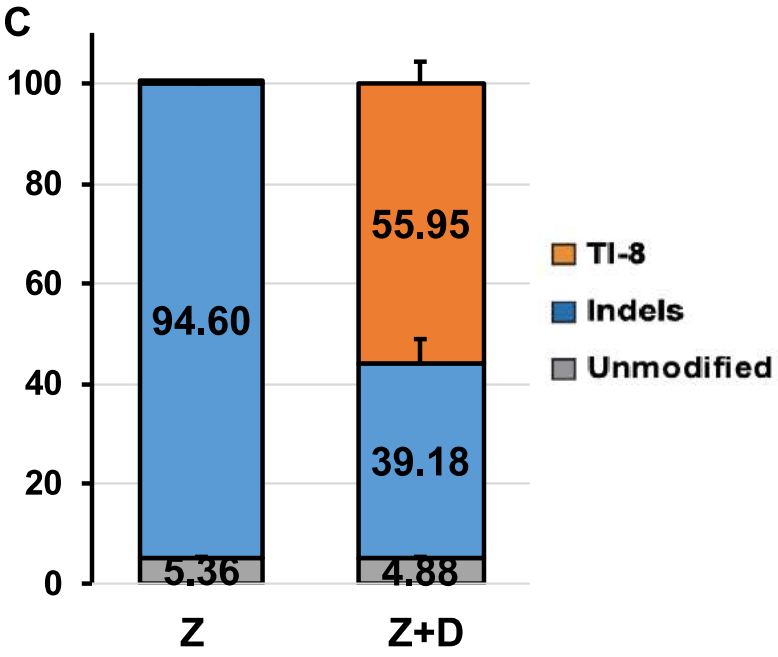
A

OT#	Mock		Parent ZFNs			Improved ZFNs			Genome notes	Locus
	%Indels	#Seq reads	%Indels	Bonferroni P-value	#Seq reads	%Indels	Bonferroni P-value	#Seq reads		
On	0.0495	54497	97.6483	0	54131	97.8171	0	48835	*CFTR* intron 8 (on-target)	chr7:117541450-117541490
OT2	0.097	57721	9.7478	0	54966	0.293	9.466E-11	40612	Intergenic	chr18:1439142-1439182
OT3	0.2061	61150	1.8446	0	42666	0.2268	0.907	52465	Intergenic	chr11:116695570-116695610
OT4	0.0367	48996	0.6216	0	38929	0.0725	0.06811	34467	Intergenic	chr1:200488808-200488848
OT10	0.0653	52075	0.4425	0	42263	0.1016	0.07636	56127	*ASCL5* intron 1	chr1:201126946-201126986
OT12	0.0087	57288	0.4717	0	39004	0.1295	1.462E-10	39391	*SARS* intron 1	chr1:109214368-109214408
OT19	0.0726	23422	0.8946	0	17214	0.2504	0.00001905	19168	Intergenic	chr17:38257580-38257620
OT21	0.032	40583	0.2062	6.217E-15	50439	0.0422	0.9376	35529	*MTNR1A* intron 1	chr4:186554568-186554608
OT25	0.0224	44568	0.1152	9.259E-07	39939	0.0569	0.01642	47421	Intergenic	chr9:97789720-97789760
OT28	0.0484	80520	0.1754	2.219E-12	67858	0.0678	0.3134	53059	Intergenic	chr1:26966994-26967034
OT32	0.1038	85763	0.2097	0.00000141	62480	0.0657	1	71526	Intergenic	chr21:43502506-43502546

B

	Exp	%TI	%Indels	%Unmodified	#Seq reads
ZFN (2ug each)	1	0.00	94.55	5.45	43320
	2	0.00	94.57	5.43	49665
	3	0.11	94.69	5.20	51279
	mean±SD	0.0 ± 0.1	94.6 ± 0.1	5.36 ± 0.1	
ZFN (2ug each) AAV6 (MOI=1x10^6)	1	51.30	44.53	4.17	45639
	2	56.62	37.91	5.47	53656
	3	59.92	35.08	5.00	47649
	mean±SD	56.0 ± 4.4	39.2 ± 4.9	4.9 ± 0.7	

C



Supplemental Figure Captions

Fig. S1. Feeder-free expansion of primary airway basal cells. (A) Population doublings (PDs) for non-CF basal cells in two established airway basal cell culture conditions, PneumaCult™-Ex Plus Medium (P-ExPlus) and dual SMAD inhibition condition (SMADinh). Non-CF basal cells at passage 1 (p1) were plated with each media and each dot represents total PD from p1 to each passage (up to p12). **(B)** Flow cytometric analysis of cell surface markers CD49f and NGFR at the indicated passage numbers. **(C)** Immunofluorescence staining of acetylated tubulin (ACT) at 4 weeks of ALI culture at the indicated number of passages. Representative 40x images in whole mount staining are shown. Scale bar: 50μm. **(D)** Representative tracings (short circuit current) of cell monolayers at 4 weeks of ALI evaluated by Ussing chamber analysis; the passage numbers are as indicated. **(E)** Summary of CFTR chloride current stimulated by forskolin and inhibited by CFTR inhibitor-172 (CFTR inh-172) is plotted. Difference of Isc (Δlsc) ($\mu A/cm^2$) before and after stimulation or inhibition of CFTR are calculated from tracings represented by (D). (mean ± SD, n=4 technical replicates)

Fig. S2. Sequence-specific correction of ΔF508 mutation. (A) The ZFN11 left (L) and right (R) pair is designed to identify and cleave ΔF508 *CFTR* sequences in exon 11. ZFN-L targets ΔF508 (red box) while ZFN-R recognizes both *wt* and ΔF508 *CFTR*. ZFN recognition sequences are colored in orange for ZFN11-L and in blue for ZFN11-R (the nucleotides within ZFN11-L and ZFN11-R that are denoted in black are skipped bases). **(B)** The percent correction mediated by ZFN11 and ssDNA was assessed by the TIDER (Tracking of Insertions, DEletions and Recombination events) bioinformatics tool (https://tider.deskgen.com/) [1]. 2μg of each ZFN11 mRNA was co-electroporated with the 200-mer ssDNA. Increases in the correction efficiency were observed with increased amounts of ssDNA. **(C)** Total cell number (x 10^6) at 3 days post-electroporation for the experiment shown in panel B. **(D)** The percent correction mediated by ZFN11 and AAV-6 donor was assessed by TIDER. Transduction with 2 kb AAV-6 donor was performed immediately after electroporation of 2μg or 3μg of each ZFN11 mRNA. Correction efficiency plateaued for MOI=10^6 (10^6 vg/cell) or higher (mean ± SD, n=3 biological replicates).

Fig. S3. Gene editing methodology does not alter airway basal cell differentiation. Immunofluorescence staining for airway epithelium markers in ALI culture. ALI cultures were established from unmanipulated cells (ΔF508/ΔF508) or manipulated cells (Z+D(AAV)) in Fig. 2B. Major epithelial cell types were identified with markers: P63 for basal cell; MUC5AC for secretory cells; FOXJ1 for ciliated cells; FOXI1 for ionocytes. Representative 40x images in transverse section staining from three random fields in each biological replicate, experiment 1, 2 and 3. Scale bar: 50μm (Panels). % of indicated cell type (mean ± SD, n=3 biological replicates). ns, not significant (Two-tailed paired t-test) (Graphs).

Fig. S4. Sequence-specific ΔF508 correction restores fully glycosylated CFTR protein expression. Western blot of protein lysates harvested at 4 weeks of ALI culture of ΔF508/ΔF508 cells that were edited with ZFN11 together with either ssDNA or AAV-6 donors. Calnexin: loading control. The mature fully-glycosylated CFTR protein is identified as band C. For ZFN/ssDNA correction of ΔF508 (% correction: 10.6 ± 2.6) the level of restored CFTR band C was 68.2 ± 16.3% of total CFTR protein. For ZFN/AAV correction of ΔF508 (% correction: 31.0 ± 4.0) the level of restored CFTR band C was 95.3 ± 5.3% of total CFTR protein. We note that since the level of band B for ΔF508/ΔF508 cells is very low (likely reflecting degradation and absence of maturation), even modest rates of correction result in the fully glycosylated band C being the main form of CFTR protein.

Fig. S5. Efficient *SA-CFTR*$_{9\text{-}27}$*-pA* TI into *CFTR* intron 8 of ΔF508/ΔF508 airway basal cells. (A) Inside-outside PCR amplification at the 5' end of the targeted transgene at 4 days of genome editing. A 788 bp PCR fragment was amplified with one primer targeting inside of the codon-optimized sequence (blue arrow in the schematic) and another primer targeting outside of left homology arm (H_L) (Black). **(B)** Indel and TI-8 diagram showing *CFTR* intron 8 genomic organization. Horizontal arrows indicate the oligo priming sites, and expected PCR amplicon sizes are shown. Only when ZFN8 and AAV-6 donor were co-delivered did Eco RI cleave the 3' end 447 bp PCR amplicon, evidence for TI-8. **(C)** Representative tracings (Isc) of cell monolayers from 5 experimental conditions: DMSO-treated, VX-

809/VX-770 pre-treated, TI7: Z7+D-treated and TI8: Z8+D-treated CF (ΔF508/ΔF508), and non-CF evaluated by Ussing chamber analysis at 4 weeks of ALI culture.

Fig. S6. Efficient *SA-CFTR*$_{8\text{-}27}$*-pA* TI into *CFTR* intron 7 of ΔF508/ΔF508 airway basal cells. (A) Schematic of site-specific targeted editing of *CFTR* intron 7. Indel diagram shows *CFTR* genomic organization between exon 7 and 8 (black boxes) (not to scale). TI-7 diagram shows intron 7 TI of human codon optimized *CFTR*$_{8\text{-}27}$ cDNA preceded by a splice acceptor, followed by bovine growth hormone (bGH) pA sequence, and flanked by 246 bp homology left (H_L) and 271 bp homology right (H_R) intron 7 sequences. Horizontal arrows indicate oligos amplifying unmodified, indel or TI-7 events and used to quantify frequency of each by NGS. Lower gel shows evidence for TI-7 via Eco RI digestion. **(B)** The genome modification frequency determined by NGS for a TI-7 experiment. The efficiency was measured 4 days after the delivery of ZFNs targeting intron 7 (ZFN7) followed immediately by AAV-6 *CFTR*$_{8\text{-}27}$ cDNA donor. Z: ZFN7 alone (23,337 NGS reads), Z+D: ZFN7 and AAV-6 donor (29,843 NGS reads). **(C)** Detection of transgene *CFTR*$_{8\text{-}27}$ mRNA. Schematic of *CFTR* endogenous and transgene mRNA. RT-PCR with oligos E7 (endogenous exon 7) and T9 (transgene exon 9) showed the expected 349 bp amplicon only in the TI-7 ΔF508/ΔF508 sample, while PCR amplification with oligos E7 and E9 (endogenous exon 9) shows the expected 349 bp amplicon in all samples. **(D)** Restoration of fully glycosylated CFTR protein via TI-7. Western blot of protein lysates harvested at 4 weeks of ALI culture. CFTR band C is absent in ΔF508/ΔF508 cells, but restored for both TI-8 and TI-7 ΔF508/ΔF508. Calnexin: loading control. **(E)** Summary of CFTR function. Bulk TI-7 ΔF508/ΔF508 cells in ALI cultures showed the restoration of CFTR function measured as Δlsc ($\mu A/cm^2$) at levels similar to ΔF508/ΔF508 cells treated with VX-809/VX-770.

Fig. S7. Efficient TI of *SA-CFTR*$_{9\text{-}27}$*-pA* into *CFTR* intron 8 of ΔF508/R553X and G542X/R785X airway basal cells. The expected inside-outside PCR amplification at the 5' end **(A)** and Eco RI digestion of the 3' end PCR fragment **(B)** was present only in TI-8 ΔF508/R553X and TI-8 G542X/R785X basal cells. **(C, D)** Representative tracings (Isc) of cell monolayers with (C) ΔF508/R553X or (D) G542X/R785X *CFTR* genotypes. The following chronic treatments and experimental conditions were applied: DMSO-treated CF, VX-809/VX-770 pre-treated CF , or TI8: Z8+D-treated CF; a non-CF control was evaluated in parallel. Ussing chamber analysis was performed at 4 weeks of ALI culture.

Fig. S8. The majority of *CFTR* transcripts from TI-8 alleles incorporates the corrective transgene sequences. (A) Schematic of *CFTR* gene showing location of PCR primers employed for genotyping of edited single-cell derived clones. Primers are identified by black horizontal arrows. **(B)** Shown is the EcoRI digestion pattern for four homozygous TI-8 clones (c6, 9D, 9H and 11G), one non-TI clone (11H), and the parental ΔF508/ΔF508 cells. TI-8 is evidenced by EcoRI cleavage of the 447 bp PCR amplicon (primers 34/35); absence of the undigested 447bp band in the EcoRI treated lane is consistent with TI-8 homozygosity. **(C)** Homozygosity of the four clones (c6, 9D, 9H, 11G) was confirmed by PCR amplification (primers Int7/Int9): the presence of the 8.64 kb band is characteristic of TI-8 while the absence of a band at 4,99 kb (or similar size due to possibility of Indels) confirms TI-8 homozygosity. **(D)** Composition of transcripts from TI-8 alleles. Shown above are schematics of normal and alternate splicing of *CFTR* transcripts. The E8/E9-10 RT-PCR amplicon can either arise from a non-TI allele (unmodified or with indels) or from alternate splicing in a TI-8 allele from the endogenous exon 8 across the corrective transgene to the downstream endogenous exon 9. The E8/T9-10 RT-PCR amplicon reflects the desired splicing from endogenous exon 8 to the transgene exon 9. The reverse primer T9-10 recognizes only codon-optimized transgene at exon 9-10 junction (blue) while the reverse primer E9-10 recognizes the endogenous exon 9-10 junction (black). Either primer together with the forward primer E8 amplify a 139 bp RT-PCR amplicon used for absolute quantitative PCR. The graph shows the composition (E-E vs. E-T) of *CFTR* transcripts.

Fig. S9. Optimization of ZFN8 decreases off-target activity while maintaining efficient cleavage and TI at intron 8. (A) Examination, via deep sequencing, of previously identified off-target sites in basal cells treated with either parental or optimized ZFN8. Ten loci out of 31 candidates originally identified via unbiased genome-wide oligonucleotide capture in K562 cells (Table S4) yielded a statistically significant (Bonferroni P-value <0.05) level of

indels in ΔF508/ΔF508 basal cells electroporated with parental ZFN8 and only 4 loci yielded a statistically significant level of indels in optimized ZFN8 as compared to mock electroporated control basal cells. **(B and C)** Frequency of genome modification in ΔF508/ΔF508 basal cells utilizing the optimized ZFN8s followed by AAV-6 transduction of the *SA-CFTR*$_{9\text{-}27}$*-pA* donor. Editing events were categorized as corrected (TI-8), indels, or unmodified. Three individual experiments were performed (mean ± SD, n=3 biological replicates); mean values are presented in (C).

Table S1

	Exp	%Corrected	%Indels	%Unmodified	#Seq reads
ZFN (2μg each)	1	0.0	44.8	55.2	30662
	2	0.0	42.1	57.9	28668
	3	0.0	47.0	53.0	35480
	mean±SD	**0.0 ± 0.0**	**44.6 ± 2.4**	**55.4 ± 2.4**	
ZFN (2μg each) ssDNA (20μg)	1	13.0	53.0	34.1	19184
	2	11.1	47.3	41.7	24706
	3	7.9	52.8	39.3	28720
	mean±SD	**10.6 ± 2.6**	**51.0 ± 3.2**	**38.4 ± 3.9**	

	Exp	%Corrected	%Indels	%Unmodified	#Seq reads
ZFN (3μg each)	1	0.0	48.9	51.1	23589
	2	0.0	44.2	55.8	28238
	3	0.0	37.4	62.5	36707
	mean±SD	**0.0 ± 0.0**	**43.5 ± 5.7**	**56.5 ± 5.7**	
ZFN (3μg each) AAV (MOI=2x10^6)	1	34.9	20.2	45.0	31866
	2	31.2	21.5	47.3	23111
	3	26.9	17.6	55.4	30344
	mean±SD	**31.0 ± 4.0**	**19.8 ± 1.9**	**49.3 ±5.5**	

Table S2

A

	#inserts	Cell type		Forskolin		CFTR Inh.-172	
				ΔIsc(mean±SD)	%non-CF	ΔIsc(mean±SD)	%non-CF
	n=4	non-CF	no treatment	62.1 ± 12.5	100	-72.2 ± 12.4	100
	n=3	ΔF508/ΔF508	DMSO	3.6 ± 0.1	5.8	-2.6 ± 0.4	3.6
	n=4		VX809/VX770	13.2 ± 2.0	21.3	-19.2 ± 3.4	26.6
Exp. 1	n=4		ZFN+ssDNA	8.6 ± 0.9	13.8	-12.4 ± 1.8	17.2
Exp. 2	n=4		ZFN+ssDNA	9.9 ± 2.0	15.9	-13.9 ± 3.1	19.3
Exp. 3	n=4		ZFN+ssDNA	6.0 ± 1.0	9.7	-7.5 ± 0.6	10.4

	#inserts	Cell type		Forskolin		CFTR Inh.-172	
				ΔIsc(mean±SD)	%non-CF	ΔIsc(mean±SD)	%non-CF
Exp. 1	n=4	ΔF508/ΔF508	DMSO	1.0 ± 0.1	1.9	-0.6 ± 0.3	1.0
	n=4		VX809/VX770	12.3 ± 3.1	23.5	-15.8 ± 3.5	27.5
	n=4		ZFN+AAV	18.9 ± 0.6	36.1	-27.4 ± 1.4	47.7
Exp. 2	n=4	non-CF	no treatment	53.4 ± 1.9	100	-55.8 ± 9.0	100
	n=4	ΔF508/ΔF508	DMSO	1.1 ± 0.1	2.1	-0.4 ± 0.7	0.7
	n=4		VX809/VX770	13.9 ± 3.6	26.0	-17.9 ± 4.9	32.1
	n=4		ZFN+AAV	24.2 ± 1.1	45.3	-32.2 ± 2.8	57.7
Exp. 3	n=4	non-CF	no treatment	44.9 ± 3.4	100	-59.7 ± 7.7	100
	n=4	ΔF508/ΔF508	DMSO	1.3 ± 0.2	2.9	-0.1 ± 1.0	0.2
	n=4		VX809/VX770	13.7 ± 1.0	30.5	-20.6 ± 0.7	34.5
	n=4		ZFN+AAV	17.6 ± 0.8	39.2	-26.0 ± 1.4	43.6

B

#Biological replicates	Cell type		Forskolin		CFTR Inh.-172	
			ΔIsc(mean±SD)	%non-CF	ΔIsc(mean±SD)	%non-CF
n=1	non-CF	no treatment	62.1	100	-72.2	100
n=1	ΔF508/ΔF508	DMSO	3.6	5.8	-2.6	3.6
n=1		VX809/VX770	13.2	21.3	-19.2	26.6
n=3		ZFN+ssDNA	8.2 ± 2.0	13.2 ± 3.2	-12.4 ± 1.8	15.6 ± 4.6

#Biological replicates	Cell type		Forskolin		CFTR Inh.-172	
			ΔIsc(mean±SD)	%non-CF	ΔIsc(mean±SD)	%non-CF
n=3	non-CF	no treatment	50.2 ± 4.7	100.0 ± 0.0	-57.5 ± 4.6	100.0 ± 0.0
n=3	ΔF508/ΔF508	DMSO	1.1 ± 0.2	2.3 ± 0.5	-0.4 ± 0.2	0.6 ± 0.4
n=3		VX809/VX770	13.3 ± 0.9	26.7 ± 3.6	-18.1 ± 2.4	31.4 ± 3.6
n=3		ZFN+AAV	20.2 ± 3.5	40.2 ± 4.7	-57.7 ± 2.0	49.6 ± 7.3

Table S3

A

	Exp	ZFN	AAV(MOI)	%TI	%Indels	%Unmodified	#Seq reads
Z	1	2µg each	NA	0.0	86.9	13.1	8061
	2			0.0	92.4	7.6	8792
	3			0.0	77.8	22.2	17385
	4			0.0	90.0	10	14872
	5			0.0	85.4	14.6	21852
	mean±SD			0.0 ± 0.0	86.5 ± 5.6	13.5 ± 5.6	
Z+D (AAV)	1	2µg each	$2x10^6$	47.0	42.8	10.2	8245
	2		$6x10^6$	50.1	43.2	6.7	8427
	3		$2x10^6$	60.3	23.5	16.2	16136
	4		$2x10^6$	64.2	28.3	7.6	17218
	5		$2x10^6$	60.7	27.4	11.9	19226
	mean±SD			56.5 ± 7.4	33.0 ± 9.3	10.5 ± 3.8	

B

ΔF508/ΔF508	Exp 2	%TI	%Indels	%Unmodified	#Seq reads
ZFN (2µg each)	4 days	0.0	92.4	7.6	8792
ZFN (2µg each) AAV6 (MOI=$6x10^6$)		50.1	43.2	6.7	8427
ZFN (2µg each)	30 days ALI	0.0	94.0	6.0	8805
ZFN (2µg each) AAV6 (MOI=$6x10^6$)		43.9	47.3	8.9	9675

C

ΔF508/R553X	Exp	%TI	%Indels	%Unmodified	#Seq reads
ZFN (2µg each) AAV6 (MOI=$2x10^6$)	1	44.9	36.2	18.9	7964
	2	48.6	20.5	30.9	16218
	3	56.6	24.8	18.6	17894
	mean±SD	50.0 ± 6.0	27.2 ± 8.1	22.8 ± 7.0	

D

G542X/R785X	Exp	%TI	%Indels	%Unmodified	#Seq reads
ZFN (2µg each) AAV6 (MOI=$2x10^6$)	1	53.2	34.8	12.0	8395
	2	66.0	25.5	8.5	14035
	3	61.7	26.2	12.0	21710
	4	66.0	26.0	7.92	17329
	mean±SD	61.8 ± 6.0	28.1 ± 4.4	10.1 ± 2.2	

Table S4

OT#	Chromosome	Start(hg38)	End(hg38)	Oligo capture events
OT1	chr7	117541450	117541490	624
OT2	chr18	1439142	1439182	400
OT3	chr11	116695570	116695610	157
OT4	chr1	200488808	200488848	154
OT5	chr7	43884378	43884418	22
OT6	chr2	172021700	172021740	21
OT7	chrX	156024044	156024084	20
OT8	chrY	57210564	57210604	20
OT9	chr1	186506	186546	19
OT10	chr1	201126946	201126986	19
OT11	chr12	16100	16140	19
OT12	chr1	109214368	109214408	18
OT13	chr15	101974938	101974978	17
OT14	chr16	15668	15708	16
OT15	chr2	30481092	30481132	16
OT16	chr4	39348778	39348818	16
OT17	chr9	16096	16136	16
OT18	chr1	15984	16024	15
OT19	chr17	38257580	38257620	15
OT20	chr17	47530832	47530872	15
OT21	chr4	186554568	186554608	14
OT22	chr2	113597412	113597452	12
OT23	chr6	13274234	13274274	12
OT24	chrX	101803712	101803752	12
OT25	chr9	97789720	97789760	12
OT26	chr12	54698438	54698478	11
OT27	chr17	38677118	38677158	10
OT28	chr1	26966994	26967034	9
OT29	chr3	90645844	90645884	7
OT30	chr3	90645930	90645970	7
OT31	chr6	5711448	5711488	7
OT32	chr21	43502506	43502546	7

Table S5

Primary antibody

Target	Host	Cat#	Manufacturer	Purpose	Dilution
CFTR	Mouse IgG2b	A4, 596	Cystic Fibrosis Foundation	Western Blot	1:1000
Calnexin	Rabbit polyclonal	ab22595	Abcam	Western Blot	1:5000
FOXJ1	Mouse monoclonal IgG1	14996580	Invitrogen	IF	1:200
FOXI1	Rabbit polyclonal IgG	HPA071469	Sigma	IF	1:200
Acetylated Tubulin	Mouse monoclonal IgG2b	T7451	Sigma	IF	1:1000
TP63 (N2C1)	Rabbit polyclonal IgG	GTX102425	GeneT ex	IF	1:100
TP63 (4A4)	Mouse monoclonal IgG2a/kappa	CM163A	Biocare	IF	1:100
MUC5AC (45M1)	Mouse monoclonal IgG1-kappa	MS-145-PO	Thermo	IF	1:200
MUC5AC (E309I)	Rabbit monoclonal IgG	61193	CST	IF	1:200
Keratin 5 (Poly19055)	Rabbit polyclonal	905501	Biolegend	IF	1:200
Keratin 5 (D4U8Q)	Rabbit monoclonal IgG	25807	CST	IF	1:200
CD49f, PE	Rat monoclonal IgG2a, κ	313612	Biolegend	Flow Cyt	1:50
CD271 (NGFR), APC	Mouse monoclonal IgG1	345108	Biolegend	Flow Cyt	1:50

Secondary antibody

Name	Cat#	Manufacturer	Purpose	Dilution
HRP-linked horse anti-mouse IgG	7076S	CST	Western Blot	1:5000
HRP-linked goat anti-rabbit IgG	ab205718	Abcam	Western Blot	1:20000
Alexa Fluor Plus 555 Donkey anti-Rabbit IgG (H+L)	A32794	Invitrogen	IF	1:500
Alexa Fluor 488 Donkey anti-mouse IgG (H+L)	A21202	Invitrogen	IF	1:500
Alexa Fluor 555 Goat anti-Mouse IgG (H+L)	A21424	Invitrogen	IF	1:500
Alexa Fluor 488 F(ab')2-Goat anti-Mouse IgG (H+L)	A11017	Invitrogen	IF	1:500

Table S6

Name	5' to 3'	purpose
CFaav2kbFw	ATAAGAATGCGGCCGCCCTCTGCTACCTCCTTTCCTT	2kb CFTR AAV6 construct
CFaav2kbRv	ATAAGAATGCGGCCGCATCTAATCCACGGTTTGCCC	2kb CFTR AAV6 construct
CFi10aFw	AGTCTATATTTGTTTTCCAGTGGC	Amplification of targeted region
CFi11aRv	TCCGCAACTTTTCCACTCGTA	Amplification of targeted region
CF5	CATTCACAGTAGCTTACCCA	Sanger sequencing
Miseq-e11-Fw	GGGAGAACTGGAGCCTTCAG	MiSeq Exon11
Miseq-e11-Rv	GTAGTGTGAAGGGTTCATATGC	MiSeq Exon11
CF34f	GCAAGGCAAGGACCAGGC	EcoR1 TI-8
CF35r	GCCAAGCACTAGGATTCATCAT	EcoR1 TI-8
CF36f	GGATGGTGTCAATATGGGTTATG	EcoR1 TI-7
CF37r	CTCTGATATCCTTGTCATCACCC	EcoR1 TI-7
72CF-wtE8f	GAAGGCAGCCTATGTGAGATAC	RT-PCR TI-8
81CF-wtE9r	CAATGTCTTATATTCTTGCTTTTGTA	RT-PCR TI-8
81CF-optE9r	GTACTCCTGCTTCTGCAGGAAGT	RT-PCR TI-8
70CF-wt7f	AGCTGGGAAGATCAGTGAAAG	RT-PCR TI-7
81CF-wtE9r	CAATGTCTTATATTCTTGCTTTTGTA	RT-PCR TI-7
81CF-optE9r	GTACTCCTGCTTCTGCAGGAAGT	RT-PCR TI-7
Miseq-i8-Fw (CF34f)	GCAAGGCAAGGACCAGGC	MiSeq intron 8
Miseq-i8-Rv	GAAGGGCTCTATTAGAGACTCC	MiSeq intron 8
Miseq-i7-Fw	GAGGTACCATTTTGGATGGTG	MiSeq intron 7
Miseq-i7-Fw	CAGGTGAGCAATAATGTTTGGG	MiSeq intron 7
CF41f	AGAGGTTGCAAATGGTGTCC	Clone genotype
CFintron7f	GAGTCCCTCTTAGTTCTGCAC	Clone genotype
CFintron9r	GTCCAGGTGCTAACAAAACTCAG	Clone genotype
WTex8 qPCRf	TATGACTCTCTTGGAGCAATAAAC	Quantitative RT-PCR TI-8
WTex9-10 qPCRr	ATTCCCCAAATCCCTCCTCC	Quantitative RT-PCR TI-8
optex9-10r	ACTCTCCGAAGCCTTCCTCC	Quantitative RT-PCR TI-8

Table S7

index	fragment ends	sample name	number of mapped read pair
AH756	paired end sequencing	Non-CF ALI	46271019
AH757	paired end sequencing	CF ALI	45444368
AH758	paired end sequencing	TI-8 CF ALI	37920997
AH774	paired end sequencing	Non-CF ALI	43663380
AH775	paired end sequencing	CF ALI	40605607
AH776	paired end sequencing	TI-8 CF ALI	29903855

Supplemental Table Captions

Table S1. Sequence-specific correction of ΔF508 mutation. Percentage of genome modification analyzed by NGS. Editing events were categorized as corrected, indels, or unmodified. Three individual experiments were performed (mean ± SD, n=3 biological replicates) and mean values are presented in Fig. 2B.

Table S2. Sequence-specific *Δ*F508 correction restores CFTR function. (A) CFTR chloride current stimulated by forskolin and inhibited by CFTR inhibitor-172 (CFTR inh-172). Mean values are obtained from 3 to 4 inserts as indicated (mean ± SD, n=3 or 4 technical replicates). **(B)** The average of indicated biological replicates, experiment 1, 2 and 3 in (A) (mean ± SD, n=3 biological replicates). Bottom half of table is presented in Fig. 2F.

Table S3. Efficient TI of *SA-CFTR*$_{9\text{-}27}$*-pA* into *CFTR* intron 8 in CF airway basal cells

Frequency of intron 8 genome modification in ΔF508/ΔF508 (A, B), ΔF508/R553X (C), and G542X/R785X basal cells (D) as determined by NGS. Editing events were characterized as corrected, indels, or unmodified. Mean and SD are shown where the biological experiments were independently replicated (n=3 to 5).

Table S4. Unbiased identification of potential off-target genome modification sites by ZFN8. A panel of 31 candidate top off-target sites (designated "OT2" through "OT32") was identified via unbiased oligonucleotide capture studies of the lead pair of ZFNs targeting human *CFTR* intron 8 in K562 cells. The top scored site of oligonucleotide capture was the intended on-target site within *hCFTR* intron 8 (hg38 coordinates chr7:117541450-117541490), which was designated "OT1".

Table S5. List of antibodies.

Table S6. List of oligos.

Table S7. Number of sequencing reads in ATAC-seq experiments.

Donor DNA sequences

200-mer ssODN (5'-3'): Site-specific ΔF508 correction

tcagagggtaaaattaagcacagtggaagaatttcattctgttctcagttttcctggattatgcctggcaccattaaagaaaatatc
atctttggtgtttcctatgatgaatatagatacagaagcgtcatcaaagcatgccaactagaagaggtaagaaactatgtgaaaact
ttttgattatgcatatgaacccttca

2 kb *wtCFTR* AAV donor DNA (From 5'ITR through 3'ITR): Site-specific ΔF508 correction

cctgcaggcagctgcgcgctcgctcgctcactgaggccgcccgggcgtcgggcgacctttggtcgcccggcctcagtgagcgagcga
gcgcgcagagagggagtggccaactccatcactaggggttcctgcccatggatttaaattctagaaggcctgcggccgcatctaatc
cacggtttgccctttccatttttttggatactgtattttaagctacattttactttctctgcaatttttttcataaaagattatat
aaaggaattgcaaatgccaactatcaaagatattgcttttgaatcacaaacactatttggagtatactgtcaataaacttcatagta
acatattccctgccctaacatttacagcaataactactgaacccaccatcacacaatttacatagtgaacagcacaagatccatata
ctggtttctcttggaatacttcatgtaattttcagataattagctcattctccaatattgttgtgaaatgagtaatgacatcatctt
catttgcctctgcatcaaagaattgcagtcactagaagttatatggtatttgttcaaagccagggatacaatatcttcacaatttt
acccctctaattctctgctggcagatcaatgctcattccattaggctatagtattattaaaattattaaaataatcttaataatttt
tggagtatatttttaaagatgcatattttgtggtatcttttaaaaagataccacatatcacttatatgcatgccatataaataacca
ttgaggacgtttgtctcactaatgagtgaacaaaattctcaccatttcataaaatgcatttattgtgatcaaatgaacccattattt
aaaaaataaattgcatttatttcatgtgtttgcaagcttcttaaagcataggtcatgtgttttattaattgatccattcacagtagc
ttacccatagaggaaacataaatatatgtagactaaccgattgaatatggagccaaatatataatttgggtagtgtgaagggttcat
atgcataatcaaaaagttttcacatagtttcttacctcttctagttggcatgctttgatgacgcttctgtatctatattcatcatag
gaaacaccaaagatgatattttctttaatggtgccaggcataatccaggaaaactgagaacagaatgaaattcttccactgtgctta
attttaccctctgaaggctccagttctcccataatcaccattagaagtgaagtctggaaataaaacccatcattattaggtcattat
caaatcacgctcaggattcacttgcctccaattatcatcctaagcagaagtgtatattcttatttgtaaagattctattaactcatt
tgattcaaaatatttaaaatacttcctgtttcaggtactctgctatgcacaaaagatacaagggaaagtaaaagagacaattacaat
acagtgtgacaagtattatgatagaggttcacagagaaggggcacatgattcagatagttggaataggtttggaggaagaatgggaa
aggttagtaacagctaacagttgccaagtgctcactctgtgtcgagtgctgttctatgtgctttaactatattaatttatttaatct
tcacagaaatcctacaaagtagattaccttcatattattaggtacagattaagtaatagagacatattcaggtaatattgttcccat
gagcctttcttggagtataaagtcatttaagagatgatagtacaaaaggactatcagtgaacaaagatttatataactgtgaacaaa
aattaaaactaatggcagaattcgagttgaaaaacaaagtttaaatatgttatatgtcctgacaataagaaaagttagaagtaaaaa
ccaaataaacaaacaaaggaaatggggtataagtgtggagtgtaaaggaaatttgctgattgctttattaagaaaagctgaaagtca
aaaggtatcatttaaagctaataaataaagtaatagaagcataagcagatttaacaatacaaagataaatctgaaaaaagataatac
tactgactaaaactgagtagaaggaaaggaggtagcagagggcggccgcaggaacccctagtgatggagttggccactccctctctg
cgcgctcgctcgctcactgaggccgggcgaccaaaggtcgcccgacgcccgggctttgcccgggcggcctcagtgagcgagcgagcg
cgcagctgcctgcagg

hCFTR_intron_7_AAV donor DNA (From 5'ITR through 3'ITR): Targeted Integration intron 7

cctgcaggcagctgcgcgctcgctcgctcactgaggccgcccgggcgtcgggcgacctttggtcgcccggcctcagtgagcgagcga
gcgcgcagagagggagtggccaactccatcactaggggttcctgcggccgcacgcgtgggcaaaatataaactacagcatttctgta
gcaatgagaccatttttcttcagttgagctccatgttctacaaacttcaatcaaaaaaggttctaggagactcagtgaaagttgata
cactgttcaaggaacaaataatttcagcacatgggaatttcacagggaaaaatatactaaaaagagaggtaccattttggatggtgt
caatatgggttatgaggaattcaggctgctgagtccagtgtaggatccctgacctcttctcttcctcccacagaacagaactgaagc
ttacccggaaggccgcctacgtgcggtacttcaacagcagcgccttcttcttctccggattcttcgtggtgttcctttccgtgttgc
cttacgcgctcattaaggggatcatcctgcggaagattttcaccacgatttccttctgcatcgtgctgaggatggccgtgactcggc
agttcccgtgggcagtccagacgtggtacgattccctgggcgccatcaacaagattcaggacttcctgcagaagcaggagtacaaga
ccttggagtacaatctcactactactgaagtggtcatggagaacgtgaccgcattttgggaggaaggcttcggagagttgttcgaaa
aggccaagcagaacaacaacaacagaaagacctccaatggcgatgattccctttttcttctccaatttctcccttcttggcactccgg
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tggtcatcatgggagaactggagccttccgagggaaagattaagcactccggtcgcatcagcttttgttcccaattttcgtggatta
tgccgggaaccatcaaggaaaacattatcttcggggtgtcatacgacgagtaccgataccgcagcgtgatcaaggcctgccagctgg
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acaagatccttatcctgcacgagggctcctcctacttctacggaaccttctccgagctgcagaacctccagcccgatttcagcagca
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aggggatgcgcccgtgtcctggaccgaaacgaagaagcagtcattcaagcaaaccggagagttcggcgaaaagcggaaaaactcga
tccttaacccgattaactccatccgcaagttctcaattgtgcaaaagacacccctgcaaatgaacggcatcgaagaggactccgacg
aacctctggaacggcggctgtcgctggtgcccgacagcgagcagggagaagccatcctcccccggatttccgtgatcagcactggcc
cgactctgcaagcccggcgccggcagtccgtgctgaacctgatgactcacagcgtgaaccagggccagaacatccatcgcaagacca
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hCFTR_intron_8_AAV donor DNA (From 5'ITR through 3'ITR): Targeted Integration intron 8

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Supplemental Materials and Methods

Culture of airway basal cells: CF and non-CF airway epithelial cells, coded as KK003K (ΔF508/ΔF508 *CFTR*), KK002C (ΔF508/R553X *CFTR*), KKD023N (G542X/R785X *CFTR*) and DD023J (non-CF), were obtained from the CF cell core facility at the University of North Carolina, NC, USA. Two culture media were used for airway basal cells culture in this study. One was Pneumacult™-Ex Plus (STEMCELL technologies, Vancouver, Canada) medium; the other was dual SMAD inhibition medium consisting of SAGM™ medium (Lonza, Basel, Switzerland) supplemented with 10 µM RhoA kinase (ROCK) inhibitor Y27362 (Reagents Direct, Encinitas), 1 µM A-8301 (R&D Systems, Minneapolis, MN), 1 µM DMH-1 (R&D Systems) and 1 µM CHIR99021 (R&D Systems)[2]. In both culture conditions, basal cells were cultured on pre-coated plates with laminin-enriched 804G cell-conditioned medium (804G-CM) and placed at 37 ºC in humidified air with 5% CO_2. When cells reached 50 to 70% confluence, they were dissociated with conventional trypsinization and either split at a 1:10 ratio or utilized for gene editing and *in vitro* differentiation. The 804G cell line, a rat bladder epithelial cell line kindly provided by Dr. Hongmei Mou (Massachusetts General Hospital, Boston, MA, USA), was cultured in RPMI-1640 (Sigma Aldrich, St. Louis, MO) supplemented with 10 % Fetal Bovine Serum (FBS) (GE healthcare, Chicago, IL) and 1 % Penicillin-Streptomycin (pen-strep) (Thermo Fisher Scientific, Waltham, MA). Once cells reached confluence in a 225 cm^2 culture flask (Corning, Corning, NY), culture supernatant was replaced with 100 ml fresh medium, and collected every other day for up to 3 collections. All media obtained were filter-sterilized and stored at 4 ºC. Both basal cells and 804G cells were cryo-preserved in CryoStor® CS10 (STEMCELL Technologies) and kept frozen in liquid N_2.

Characterization of airway basal cells: Airway basal cells were analyzed with cell surface markers CD49f and NGFR using flow cytometric analysis. Briefly, dissociated cells were stained with α-CD49f-PE (Biolegend, San Diego, CA) and/or α-CD271(NGFR)-APC (Biolegend) on ice for 30min. Non-immune IgG2a-PE and IgG1, as well as κ-APC were used as isotype controls. Antibody-stained cells were washed with FACS buffer (phosphate buffered saline with 1 % FBS), pelleted by a 5-minute centrifugation at 200g and then re-suspended with FACS buffer containing 0.075 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) for live cell separation. Stained cells were analyzed on a FACS LSRII (BD Biosciences, San Jose, CA) using FACS Diva software (BD Biosciences).

***In vitro* differentiation of basal cells at air liquid interface (ALI):** 200,000 airway basal cells in Pneumacult™-Ex Plus medium were seeded on the top chamber of a 6.5 mm Transwell® with 0.4 µm pore polyester membrane inserts (Corning) pre-coated with 804G-CM. Initially, medium was added to both the top and bottom chambers. The medium was replaced the following day by Pneumacult-ALI medium (STEMCELL Technologies). After an additional day, medium from the top chamber was removed to establish the ALI and cells were maintained, with daily feeding, in this manner for approximately 4 weeks.

Histological and immunofluorescence analysis: Well-differentiated airway epithelia at ALI were fixed at 4°C overnight in 4 % paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA). Fixed samples were dehydrated with a series of increasing concentrations of ethanol, cleared with xylene and infiltrated with paraffin before embedding in wax. Sections of 5 µm thickness were transversely cut using an HM 325 Rotary Microtome (Thermo Fisher Scientific), followed by staining with haematoxylin and eosin. For immunofluorescence analysis, fixed cells were immunostained on transwell inserts to perform whole mount staining, or cryo-sectioned transversely to image airway epithelium. Cryo-section samples were prepared following sequential sucrose treatment (15 %, then 30 % sucrose), flash freezing in OCT embedding medium (Thermo Fisher Scientific), and sectioning (5-8 µm thickness) using a Leica® CM1850 Cryostat (Leica Biosystems Inc., Buffalo Grove, IL). Whole inserts and sectioned samples were stained with primary/secondary antibodies listed in **Table S5** utilizing standard immunofluorescence procedures. Briefly, samples were permeabilized with 0.3 % Triton X-100 (Sigma) in PBS for 15-30 min and blocked with 2 % bovine serum albumin (BSA) for 1 hour. Samples were then incubated with primary antibodies in 2 % BSA overnight at 4 ºC, followed by the incubation with the respective secondary antibodies in 2 % BSA at room temperature for one to two hours. Three 5 minute washes in PBS were performed after each antibody treatment. Prolong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) was added to counter-stain. Samples were then mounted and cured for 24 hours prior to imaging. Images were acquired using a Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany) and Leica Application Suite Software (Leica

Microsystems). Quantification of each epithelial cell type was performed by counting the number of cells staining for specific markers in three random 40x magnification fields. The frequency of each cell type was calculated relative to the total number of cells as determined by DAPI stained nuclei (approximately 450 cells total from three random fields). Statistical calculations in Figure S3 were performed using GraphPad Prism® v8.2.1 (GraphPad Software, San Diego, CA) and statistical significance was determined using Two-tailed paired t-test compared to control with 95% confidence interval.

Gene editing reagents: Zinc Finger Nucleases (ZFNs) targeting intron 7, intron 8, or the ΔF508 mutant sequence of human *CFTR* were subcloned into individual vectors (pVAX-GEM) containing a T7 RNA polymerase promoter, a 5' UTR sequence derived from the Xenopus beta-globin gene, a 3' UTR containing the Woodchuck Hepatitis Virus Response Element (WPRE) sequence, and polyA tract. ZFN mRNAs were synthesized *in vitro* either commercially (TriLink BioTechnologies, San Diego, CA) with ARCA Cap modification or in house using the mMESSAGE mMACHINE T7 Ultra kit followed by the purification with a MEGAclear kit (Thermo Fisher Scientific).

An approximately 2 kb *wtCFTR* AAV-6 donor spanning exon 11 was prepared by PCR amplification of *wtCFTR* (1963bp) from DD023J (non-CF) genomic DNA utilizing PCR primers CFaav2kb Fw and R, cloning into an AAV-2 ITR-containing plasmid backbone (pAAV2-MCS; Cell Biolabs, San Diego, CA), and produced commercially (Vigene Biosciences, Inc., Rockville, MD). The partial *CFTR* cDNA intron 7 and intron 8 donor constructs contained a codon-optimized human partial *CFTR* cDNA, a splice acceptor sequence derived from human FIX, and the bovine growth hormone (bGH) poly adenylation sequence. These constructs, denoted *SA-CFTR*$_{8\text{-}27}$*-pA* and *SA-CFTR*$_{9\text{-}27}$*-pA*, were flanked by homology sequences of approximately 500-600 bp in total length from human *CFTR* introns 7 and 8, respectively. To simultaneously measure gene disruption (indels) and homology directed repair (HDR)-mediated TI alleles, AAV donor constructs also included a primer binding site (green arrows in **Figure 3A** for intron 8 targeting) followed by a TI-specific barcode (gray box in **Figure 3A** for intron 8 targeting) just upstream of the right homology arm. Recombinant AAV-2/6 vectors (comprised of AAV-2 ITRs and the AAV-6 capsid) carrying intron 7 and intron 8 partial *CFTR* cDNA donors were produced by triple transfection of HEK293 cells in 10-chamber CELLSTACK culture chambers (Corning), and purified by cesium chloride density gradient centrifugation followed by dialysis. Viral genome concentrations were measured by quantitative polymerase chain reaction (qPCR). The 200-mer single strand oligo DNA donor (ssDNA) was synthesized by Integrated DNA Technologies (IDT) (Coralville, IA). All donor DNA sequences are shown above (Donor DNA sequences).

Gene editing: Trypsin-dissociated airway basal cells were resuspended in 100µl BTXpress™ solution (Harvard Apparatus, Holliston, MA) with ZFN mRNAs and electroporated in BTX™ electroporation cuvettes (2mm gap, Harvard Apparatus) under Low Voltage (LV) conditions of 250 V for 5 ms, and 1 pulse using BTX™ ECM 830 electroporation generator (Harvard Apparatus). The number of cells (2–5 x 10^5 cells per reaction) and ZFN mRNA amount (typically 2-3 ug of each ZFN-L and ZFN-R mRNA per reaction) were optimized for each targeting site and strategy. The electroporated cell/ZFN mRNA solution was transferred into a 1.5 ml tube and immediately transduced with AAV-6 donor at a multiplicity of infection (MOI) optimized for each targeting site (typically 1-6 x10^6 viral genomes per cell (vg/cell)) for 20 min. Cells, together with AAV-6 donor, were cultured overnight in Pneumacult™-Ex Plus medium on 804G CM-coated plates. Alternatively, sequence-specific correction of ΔF508 mutation with ssDNA donor was performed via co-electoroporation of 2 µg each ZFN11 mRNA together with 10 - 30 µg of 200-mer ssDNA donor. Co-electroporated cells were immediately plated in Pneumacult™-Ex Plus medium on 804G-CM-coated plates.

Assessment of on-target genome modification: Induction of on-target indels, sequence-specific correction, and targeted integration were assessed quantitatively through NGS deep sequencing on an Illumina platform. For assessing ZFN11-induced indels and ΔF508 correction, genomic DNA (gDNA) from gene-edited cells was first PCR-amplified using primers CFi10aFw and CFi11aRv indicated as f_1 and r_2 in **Figure 2A**, respectively, in order to avoid sequencing of residual ssDNA oligo or episomal AAV-6 donor DNAs. Nested PCR amplification was subsequently performed using primers Ex11 Miseq-Fw and -Rv to prepare ~ 200 bp amplicons for paired-end deep sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA).

In order to permit simultaneous NGS-mediated assessment of gene disruption (indels) and HDR-mediated TI alleles for the targeting of introns 7 and 8, AAV donor constructs also included a primer binding site followed by a TI-specific barcode just upstream of the right homology arm (highlighted in **Figure 3A** for intron 8 targeting, for example). Once amplified with a reverse primer (black arrows in **Figure 3A** for intron 8 targeting) which binds just downstream of the right homology arm within the genome, both wildtype and modified (indels and HDR-mediated TI) alleles were amplified simultaneously, and frequencies of each mode of gene modification were assessed via paired-end MiSeq sequencing. The system was designed such that no PCR bias occurs with amplification of the TI alleles vs. wildtype alleles since the PCR amplicon was the same length and base composition. Examples of sequencing data are uploaded in supplementary files (Intron 8 NGS.xlsx, Intron 7 NGS.xlsx). One TI-8 experiment in ΔF508/ΔF508 cells, showing extremely low frequency of indels as well as TI-8, was excluded from further analysis and presentation – due to concern that the electroporation step on this occasion was not successful.

In some cases, as in **Figure S2**, sequence-specific correction of ΔF508 mutation was also assessed by the web based bioinformatics tool "Tracking of Insertions, DEletions and Recombination events" (TIDER) (https://tider.deskgen.com/) [1]. Briefly, genomic DNA from edited cells was amplified by PCR with primers CFi10aFw and CFi11aRv and Sanger sequenced with primer CF5 followed by TIDER analysis. The 'guide sequence' for the TIDER analysis was 5'- AATATCATTGGTGTTTCCTA-3'; control and reference chromatogram for the analyses were sequences from genomic DNA of KK003K (Δ*F508/*Δ*F508 CFTR)* and DD023J (non-CF), respectively. TI-8 was also assessed semi-quantitatively by PCR amplification of genomic DNA from edited cells with primers CF34f and CF35r, followed by digestion with Eco RI (**Figure S5B**). TI-7 was similarly analyzed by PCR amplification with primers CF36f and CF37r, followed by Eco RI digestion. Digested and undigested PCR product were resolved with a 3 % agarose gel.

Southern blot analysis of edited cells: 20µg of genomic DNAs (gDNAs) isolated with GentraPura gene Core A (Qiagen, Hilden, Germany) were digested overnight with Eco RI and purified by ethanol precipitation. The gDNAs were then resolved on 0.7 % agarose gel, transferred to a Nytran Super Charge membrane (GE Healthcare), and hybridized with [^{32}P]-labeled probe. As probe, a 246 bp *CFTR* exon 8 fragment was synthesized (IDT) and labeled with [^{32}P]dCTP using Prime-It II Random Primer Labeling kit (Agilent Technologies, Santa Clara, CA). Following hybridization, the membrane was washed, exposed to X-ray film, and scanned.

***CFTR* RT-PCR:** Total RNA was isolated from ALI culture with the Nucleospin RNA XS kit (Macherey-Nagel Inc, Bethlehem, PA). cDNA synthesis was performed with the Improm-II Reverse Transcriptase oligo dT kit (Promega, Madison, WI) and RT-PCR were performed with Gotaq Hot Start polymerase (Promega). TI-8 cDNA samples were analyzed with PCR primer pairs 72CF-wt8f and 81CF-wt9r for the endogenous transcript and with primers 72CF-wt8f and 81CF-opt9r for the chimeric endogenous-transgene transcript. TI-7 cDNA samples were analyzed with primers 70CF-wt7f and 81CF-wt9r for the endogenous transcript and with primers 70CF-wt7f and 81CF-opt9r for the chimeric endogenous-trangene transcript.

Western blot analysis of CFTR protein: After thorough washing with PBS, ALI cultured cells were lysed with RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). Lysate was twice flash frozen and thawed, and centrifuged at 3000 x g for 10 min (4 ºC) to obtain protein extracts. Sixty µg of protein was brought to a volume of 50 µl with RIPA buffer, sample reducing agent (Thermo Fisher Scientific), and sample buffer (Thermo Fisher Scientific). Proteins were resolved by electrophoresis through a NuPAGE 7 % Tris-Acetate Protein Gel using SDS-PAGE methodology, transferred onto Hybond-C nitrocellulose transfer membrane (GE Healthcare), and blocked with 5 % non-fat dry milk in PBS (blocking buffer) at room temperature for 30 minutes. The blot membrane was incubated in blocking buffer overnight at 4 ºC with anti-CFTR primary antibody A4 596 (Cystic Fibrosis Foundation Therapeutics), followed by 4 washing steps for 5 minutes each in 1xTBS containing 0.1 % Tween 20 and 0.25 % non-fat dry milk (washing buffer). Subsequently, the membrane was incubated for 1 hour at 4 ºC in in blocking buffer containing HRP-linked horse anti-mouse secondary antibody (Cell Signaling Technologies, Danvers, MA), followed by washing 4 times with washing buffer and chemiluminescent detection using Amersham

ECL Prime (GE Healthcare). As loading control, calnexin was probed and detected with anti-Calnexin polyclonal antibody (Abcam, Cambridge, United Kingdom) followed by secondary HRP goat anti rabbit IgG (Abcam).

Ussing chamber analysis: Ussing chamber experiments were performed on EasyMount Ussing Chamber Systems at voltage clamp mode, and Acquire & Analyze software (Physiologic Instruments) was employed to record and analyze data. Briefly, transwell inserts were mounted into chambers and bathed in low chloride Ringer`s solution (1.2 mM NaCl, 140 mM Na-gluconate, 25 mM $NaHCO_3$, 3.33 mM KH_2PO_4, 0.83 mM K_2HPO_4, 1.2mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose) at apical and Ringer's solution (120 mM NaCl, 25 mM $NaHCO_3$, 3.33 mM KH_2PO_4, 0.83 mM K_2HPO_4, 1.2mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose) at basolateral side of monolayer. After base line is stabilized, 100 µM amiloride (Sigma) applied to both sides of the chamber to carry out complete inhibition of ENaC (Epithelial sodium channel). Subsequently, 10 µM forskolin (Sigma) were administered to stimulate chloride current. At the end of experiments, 10 µM CFTR inhibitor-172 (Sigma) employed at the apical side to specifically inhibit CFTR function following by 100 µM UTP at the apical side to asses the integrity of developed epithelium. The resulting change in short circuit current was calculated as ΔI_{sc}. For some samples, ALI-cultured cells were pre-treated with 3 µM VX-809 and 1 µM VX-770 for 48 hour prior to Ussing chamber analysis to modulate CFTR expression and function. The data were expressed in mean±SD.

Clonal isolation of TI-8 cells: Single cell-derived clones were isolated from bulk TI-8 treated cells via limiting dilution utilizing a modified conditionally reprogrammed cell (CRC) method[3, 4]. Briefly, bulk edited cells were dissociated with trypsin into single cells and diluted in CRC medium consisting of a 3:1(v/v) mixture of complete DMEM medium (DMEM high glucose containing 10% FBS, 2mM L-glutamine and 100U/ml Penicillin-Streptomycin) (Gibco) and F-12 Nutrient Mix (Gibco), 25 ng/ml hydrocortisone (Sigma), 0.125 ng/ml EGF (Invitrogen), 5 µg/ml insulin (Sigma), 250 ng/ml fungizone/amphotericin B (Fisher), 10 µg/ml Gentamicin (Gibco), 0.1 nM cholera toxin (Sigma) and 10 µM Y-27362 (Reagents Direct) and then plated by limiting dilution onto a feeder layer of irradiated NIH3T3 cells in a 96 well plate. Each well was monitored via inverted light microscopy for the appearance of a single colony per well. Each colony was further expanded in SMAD inhibition medium or in PneumaCult™ ExPlus medium.

Quantification of TI-8 mRNA transcript composition: Quantitative RT-PCR was performed on RNA isolated from single-cell derived clones of TI-8 edited airway basal cells that had been cultured under ALI conditions for 3 - 4 weeks. Quantitative RT-PCR was performed using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, CA) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The endogenous *CFTR* transcript was amplified with primer pair: WTex8 qPCRf (E8 in **Figure S8D**) and WTex9-10 qPCRr (E9-10 in **Figure S8D**). The chimeric endogenous-transgenic *CFTR* transcript was detected with the primer pair: WTex8 qPCRf (E8) and optex9-10 qPCRr (T9-10 in **Figure S8D**). The standard curve for absolute quantification of *CFTR* transcript copy number was obtaind from serial diliution of a 250 bp double stranded, synthesized DNA that contained either the endogenous exons 8, 9 and 10 or the endogenous exon 8 directly joined to codon optimized transgenic exons 9 and 10.

Assessment of ZFN8 off-target genome modification and ZFN8 optimization: The lead h*CFTR* intron 8 - targeted ZFN pair was subjected to unbiased identification of candidate off-target sites using methods similar to those previously described[5]. Briefly, K562 cells were electroporated with mRNA encoding the ZFNs as well as barcoded DNA oligonucleotides using the BTX ECM 830 electroporator to allow for unbiased identification of sites which had undergone double-stranded DNA cleavage and NHEJ-mediated integration of the DNA oligonucleotides. The top 32 loci containing integrated oligonucleotides was then subjected to a validation procedure in primary human basal airway epithelial cells. For each off-target site (designated "OT2" through "OT32"), an oligonucleotide primer pair was designed that enabled amplification of a 120-200 base pair (bp) fragment. Primers were also similarly designed for the intended target in *CFTR* (designated "OT1"). Primers designed against target loci were then screened for predicted amplification specificity by a genome-wide *in silico* PCR simulation. Optimal primer pairs emerging from this step were extended via appending of adapter sequences necessary for a second "barcode"

PCR to attach priming sites and barcodes for the MiSeq process. The ZFN design was optimized as described [6] in order to reduce the incidence of off-target indels, while still retaining recognition of the same intron 8 target sequence.

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