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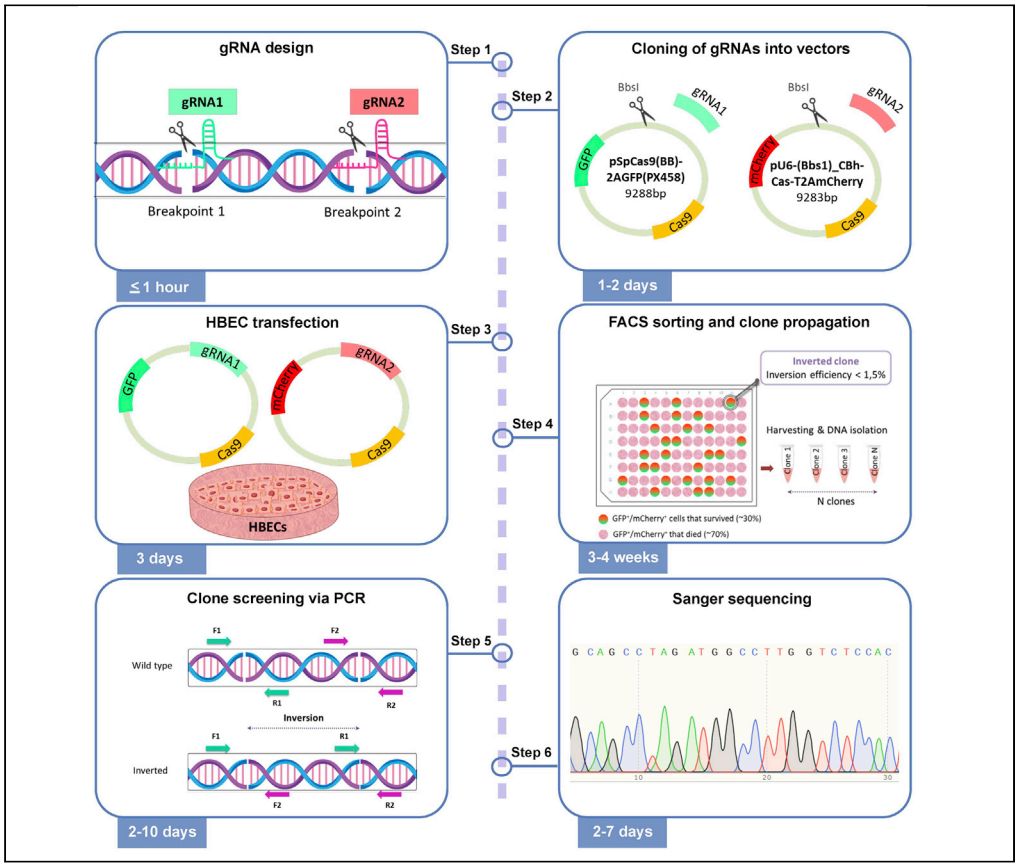
CRISPR-Cas9-mediated induction of large chromosomal inversions in human bronchial epithelial cells



The *in vitro* recapitulation of chromosomal rearrangements is a necessary tool for understanding malignancy at the molecular level. Here, we describe the targeted induction of a large chromosomal inversion (>3.7 Mbp) through CRISPR-Cas9-mediated genome editing. As inversions occur at low frequency following Cas9 cleavage, we provide a detailed screening approach of FACS-sorted, single-cell-derived clonal human bronchial epithelial cell (HBEC) cultures. The protocol provided is tailored to HBECs; however, it can be readily applied to additional adherent cellular models.

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Highlights

Large chromosomal inversions engineered with CRISPR-Cas9 technology in the HBEC system

Step-by-step protocol of gRNA design and cloning into Cas9-encoding vectors

Clonal propagation of FACS-sorted single cells following non-liposomal transfection

Description of primer design strategy and PCR screening for inversion validation

Angelopoulou et al., STAR Protocols 3, 101257
June 17, 2022

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CRISPR-Cas9-mediated induction of large chromosomal inversions in human bronchial epithelial cells

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https://doi.org/10.1016/j.xpro.2022.101257

SUMMARY

The *in vitro* recapitulation of chromosomal rearrangements is a necessary tool for understanding malignancy at the molecular level. Here, we describe the targeted induction of a large chromosomal inversion (>3.7 Mbp) through CRISPR-Cas9-mediated genome editing. As inversions occur at low frequency following Cas9 cleavage, we provide a detailed screening approach of FACS-sorted, single-cell-derived clonal human bronchial epithelial cell (HBEC) cultures. The protocol provided is tailored to HBECs; however, it can be readily applied to additional adherent cellular models.

For complete details on the use and execution of this protocol, please refer to Zampetidis et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps of inducing a targeted chromosomal inversion in human bronchial epithelial cells (HBECs).

Design of gRNAs

Timing: 0.5–1 h

In order to obtain a chromosomal inversion, two independent chromosomal breaks must be induced. Therefore, two different gRNAs are required to direct Cas9 binding and cleavage at two separate genomic positions. Specifically, each breakpoint should be the target of an individual gRNA. In our study, we aimed at recapitulating an inversion naturally occurring in cancerous cells that have escaped from oncogene-induced senescence, as identified by Whole Genome

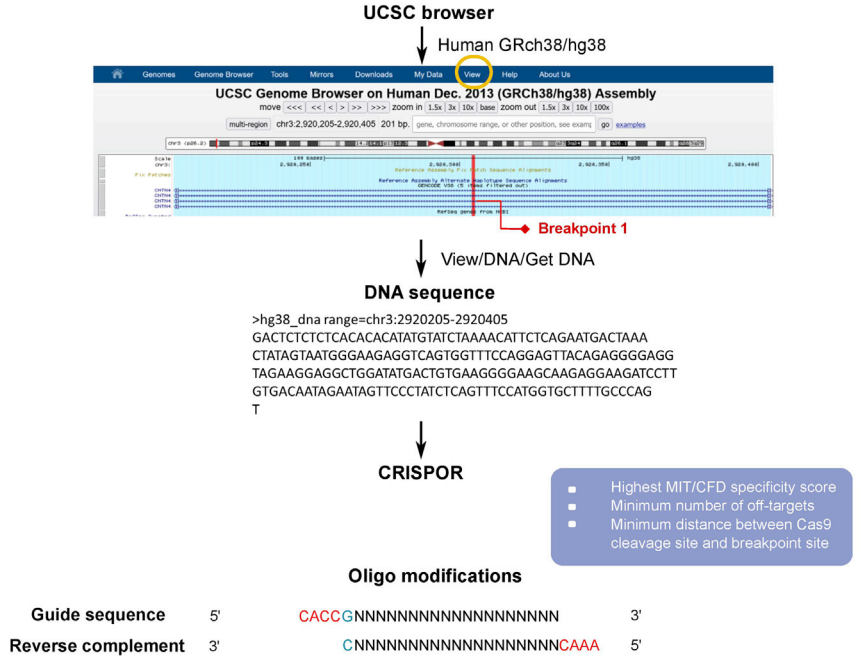

Figure 1. Flow chart of the gRNA design process using the UCSC browser and CRISPOR algorithm

Based on the breakpoint position (red highlight), the DNA sequence around it (blue highlight) can be obtained using the UCSC browser. The acquired sequence is then imported to CRISPOR serving as target for the design of gRNAs. The gRNAs predicted to have the highest specificity scores and the smallest distance from the breakpoint are chosen and modified with the proper overhangs before ordering.

Sequencing (WGS) (Zampetidis et al., 2021). Based on the precise inversion coordinates (start/end), two gRNAs are designed, one for each breakpoint, following the steps below (see Figure 1):

1. The DNA sequence used as target for the design of the two gRNAs can be obtained via the UCSC Genome Browser (https://genome.ucsc.edu/).
   a. Choose the genome version of interest. In our case, "Human GRCh38/hg38" was used.
   b. Based on the known position of inversion breakpoint 1, mark the genomic area ranging from 100-150 bp before to 100–150 bp after the breakpoint. For example, if breakpoint 1 is located at chr3:2,920,305, in the UCSC Genome Browser search box type "chr3:2,920,205–2,920,405" to mark the required chromosomal area and click on "Go".
   c. Select "View" on the UCSC Genome Browser toolbar and click on the "DNA" option.
   d. In the new window, click on "Get DNA" in order to obtain the exact DNA sequence. This is the sequence required to design gRNA primers using the CRISPOR algorithm (see step 2a, below).
   e. Repeat steps 1a–1d for breakpoint 2 of the inversion.
2. To design the gRNAs use the CRISPOR algorithm (http://crispor.tefor.net/):
   a. Enter the DNA sequence obtained from step 1d for breakpoint 1. Make sure the reference genome matches the one used in the UCSC Browser (step 1a) and subsequently select a Protospacer Adjacent Motif (PAM) that is identifiable by the type of Cas9 enzyme encoded by the transfection vector. If SpCas9 is expressed by the transfection vector, selected the 20 bp-NGG PAM format. Click "Submit" to obtain candidate gRNA sequences targeting the template DNA.
   b. The CRISPOR algorithm by default ranks candidate gRNA sequences from highest to lowest specificity, as this is the critical parameter. From the list of candidate gRNAs appearing on a new page, choose the guide sequences with the highest Massachusetts Institute of Technology (MIT) and Cutting Frequency Determination (CFD) specificity scores (Doench et al., 2016; Hsu et al., 2013; Tycko et al., 2019). These scores evaluate candidate gRNAs in terms of

potential off-target effects, with the highest MIT/CFD scores corresponding to the smallest number of potential off-target events. Among guide sequences with similar MIT/CFD scores, select the ones offering the minimum distance between the Cas9 cleavage site and the known breakpoint 1 site. Regarding the Cas9 cleavage site, SpCas9 normally induces a blunt cut between the 17th and 18th base in the target sequence (3–4 bp 5′ of the PAM) (Jinek et al., 2012).

c. Repeat steps 2a and 2b for the DNA sequence corresponding to breakpoint 2.

⚠ CRITICAL: Avoid selecting sequences that are indicated as "Inefficient" or displaying "High GC content" by the CRISPOR algorithm, as this may significantly compromise target cleavage.

Note: More than one gRNAs per each breakpoint need to be designed and tested for optimal efficiency.

3. Before ordering the gRNAs as standard desalted oligos, the selected gRNA sequences should be modified as follows:
   a. Make sure the PAM sequence is not included in the gRNA oligos to be ordered. Remove the PAM sequence from gRNAs before proceeding to the next steps.

⚠ CRITICAL: The PAM sequence is located downstream of the target DNA sequence and is essential for Cas9 binding and cleavage, but it should not be part of the gRNA oligos, to avoid unwanted Cas9-mediated cleavage of the gRNA itself.

Note: As an alternative to removing the PAM sequence from gRNAs before ordering, random nucleotide mutations may be introduced to the PAM sequence so that it is not in an NGG format. This way Cas9-mediated cleavage will be prevented.

   b. Each gRNA sequence yielded by the CRISPOR algorithm serves as the Forward (FW) gRNA sequence. Generate the reverse complement of the FW gRNA sequence to obtain the Reverse (REV) gRNA sequence. The following online tool can be used to directly obtain the reverse complement of an input sequence: https://www.bioinformatics.org/sms/rev_comp.html
   c. Add to both gRNA sequences (FW and REV) the proper overhangs in order to render them compatible for cloning into the gRNA scaffold of the transfection vector. In our experimental setup, two PX330-based plasmids were used as cloning vectors: pSpCas9(BB)-2A-GFP (PX458) (#48138; Addgene) (Ran et al., 2013) and pU6-(BbsI)_CBh-Cas9-T2A-mCherry (#64324; Addgene) (Chu et al., 2015), therefore before ordering the gRNAs we made the following nucleotide additions:
      i. CACCG to the 5′ end of the FW gRNA sequence.
      ii. AAAC to the 5′ end and C to the 3′ end of the REV gRNA sequence.

⚠ CRITICAL: Addition of G in the FW sequence and C in the REV sequence should be done only if the FW sequence does not start with G, as the initiation of transcription at the RNA Pol III U6 promoter is enhanced by the presence of a G at the transcription start site.

Design of primers for identifying the inversion

Timing: 0.5–1 h

The successful generation of the inversion can be identified by PCR using specific primer combinations (Figure 2A). Initially, two pairs of primers are designed: one pair of Forward (F1) and Reverse (R1) primers amplifying wild-type genomic DNA before the induction of breakpoint 1, and a second pair of Forward (F2) and Reverse (R2) primers amplifying wild-type genomic DNA before the

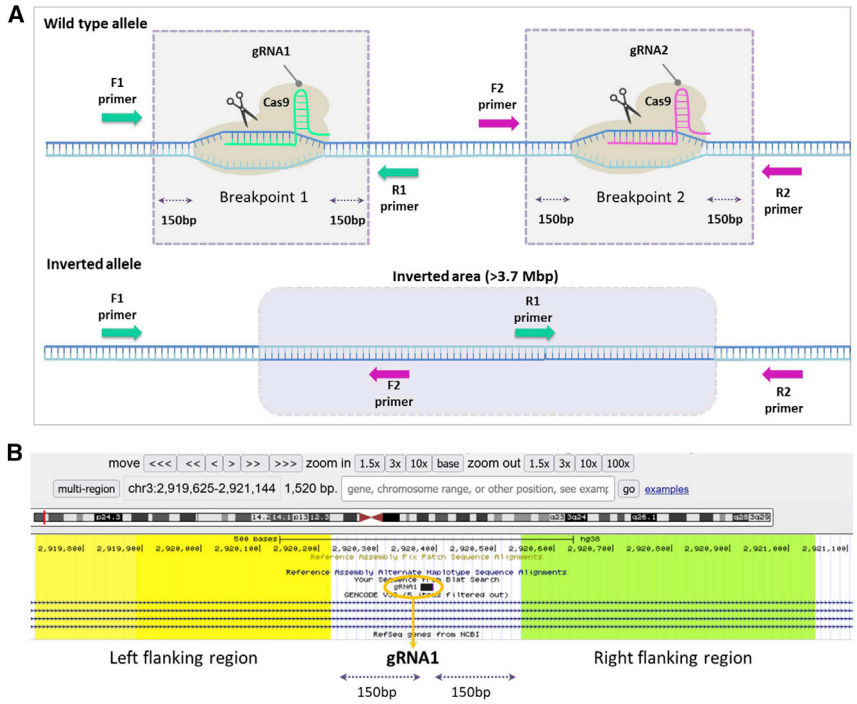

Figure 2. Primer design for identification of the inversion

(A) Schematic illustration of the position of the primers around breakpoints.

(B) UCSC browser image depicting the flanking region on the left side of the gRNA1 (yellow highlight) and on the right side (green highlight), based on which the F and R primers are designed, respectively. Flanking regions are selected at least 150 bp away from the Cas9 cleavage site to ensure proper amplification.

induction of breakpoint 2. In the case that the combination of F1/F2 or R1/R2 primers yields a PCR product, at least one allele has been inverted (Figure 2A). In detail:

4. To acquire the DNA sequence serving as template for the design of primers:
   a. Use the UCSC Genome Browser (https://genome.ucsc.edu/). Go to "Tools" on the Toolbar and Click on "BLAT" (Figure 3A).
   b. Insert the gRNA sequence in FASTA format (Figure 3B). For example, for breakpoint 1, insert the respective gRNA sequence (gRNA1) as follows:

```
``

>gRNA1

GCACCATGGAAACTGAGATA

''
```

   c. Click on "Submit" and in the new window choose "browser". This will allow visualization of the DNA sequence corresponding to gRNA1 (step 4b) (Figure 3C).
   d. As F1 and R1 primers should be located >150 bp away from the breakpoint, zoom out (by clicking on the "10×" button, Figure 3C) and select the flanking regions spanning >150 bp away from each end of the gRNA sequence (see Figure 2B).
   e. To acquire the left flanking sequence click on "View" → "DNA" and "Get DNA". This area will be used as a template to design primer F1.

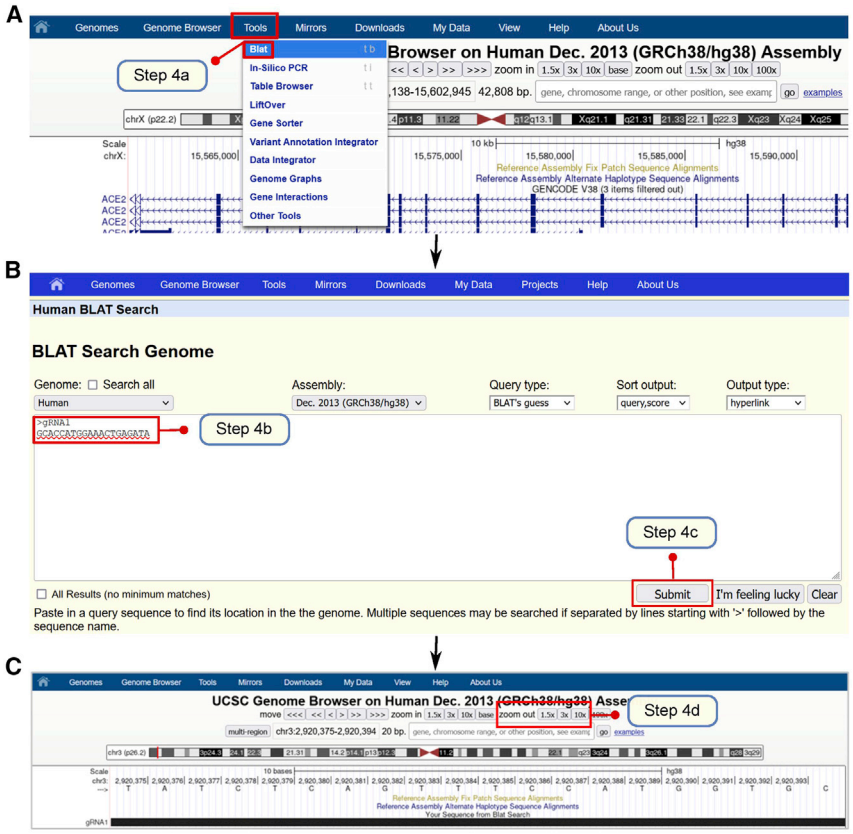

Figure 3. Step-by-step guide to acquire the DNA sequence serving as template for the design of primers

(A–C) Visualization of (A). Step 4a (B). Step 4b and (C). Step 4c of the "design of primers for identifying the inversion" section of the protocol.

f. To acquire the right flanking sequence repeat step 4e on the right side of gRNA1. This sequence will be used as a template to design primer R1.

g. Carry out the above Steps (4a–f) separately for breakpoint 2 to acquire the DNA templates to also design primers F2 and R2.

5. The primer designing tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) can be used to design the primers:

a. Enter in the "PCR template" box the left flanking sequence for gRNA1 obtained from step 4.

b. Modify the settings as follows:

Primer melting temperatures (Tm): 59°C–61°C
Database: RefSeq representative genomes
Advanced settings:
Primer size: 19–22 nt
Primer GC content (%): 45–75

c. Repeat steps 5a and 5b for the right flanking sequence of gRNA1, as well as for the two flanking sequences of breakpoint 2.

⚠ CRITICAL: Selected primers should target regions at least 150 bp away from the Cas9 cleavage site to ensure correct amplification of the desired DNA fragment containing the breakpoint. The reason is that repair of the cleaved site occurs randomly (through Non-Homologous End Joining (NHEJ)) and may variably affect the DNA sequence proximal to the cleaved site.

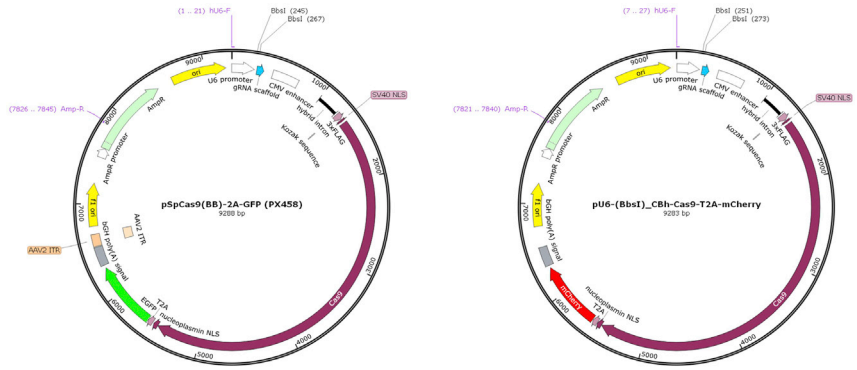

Figure 4. Empty backbones of Cas9/GFP (left) or Cas9/mCherry (right) expression vectors

Note: As per convention, F primers are derived from left flanking sequences and R primers from right flanking sequences of the gRNAs. F and R primers need to fully encompass their respective breakpoint.

Note: In the case that no primers are returned using the above settings (step 5b), the PCR template sequence can be expanded up to ~1,000 bp around each gRNA (Figure 2B).

Note: Choose more than one set of primers for each breakpoint in order to facilitate the screening process, as well as for validation purposes.

Choosing gRNA expression vectors

Two plasmid vectors, both expressing Cas9 but different fluorescent markers, are chosen for the cloning of the two gRNAs. gRNA sequences are processed in order to be able to be effectively cloned into the selected vectors, as indicated in step 3c. In our experimental setting, the gRNAs designed at steps 1–3 to target the breakpoints of the inversion were cloned separately into pSpCas9(BB)-2A-GFP (PX458) (#48138; Addgene) (Ran et al., 2013) and pU6-(BbsI)_CBh-Cas9-T2A-mCherry (#64324; Addgene) (Chu et al., 2015) (Figure 4).

Note: Fluorescent labeling of these vectors facilitates single cell isolation by FACS, as described below.

Cloning of gRNAs into vectors

Timing: 1–2 days

6. Annealing of oligos and cloning steps were based on the protocol of the Zhang lab (https://media.addgene.org/data/plasmids/62/62988/62988-attachment_i-jdFt6Gm-ft.pdf):
   a. Digest each plasmid with BbsI. The restriction cutting site of BbsI is located next to the gRNA scaffold in both plasmids. Set up the reactions as follows and incubate for 30 min at 37°C:

Component	Volume
Plasmid (1 μg)	X μl
10× NEBuffer	5 μL
BbsI (10 units)	1 μL
ddH_2O	up to 50 μL
Total	**50 μL**

b. Run the reaction in a 0.8% agarose gel and extract the digested plasmid using the Zymoclean Gel DNA Recovery Kit (https://files.zymoresearch.com/protocols/_d4001t_d4001_d4002_d4007_d4008_zymoclean_gel_dna_recovery_kit.pdf) and elute in 6 μL Elution buffer. Recipes for 0.8% and 1.3% agarose gels used in this protocol are provided in the "materials and equipment" section.
c. Phosphorylation and annealing of oligos.
   i. Set up the reaction for each pair of oligos, as follows:

Component	Volume
Oligo 1 (100 μM)	1 μL
Oligo 2 (100 μM)	1 μL
10× T4 DNA Ligase Reaction Buffer	1 μL
T4 PNK (10 units)	1 μL
ddH20	6 μL
Total	**10 μL**

   ii. Incubate at 37°C for 30 min followed by incubation at 95°C for 5 min and then ramp down to 25°C at 5°C/minute.
   iii. The phosphorylated and annealed oligos can be stored at −20°C.
d. Prepare the ligation reaction as follows and incubate for 60 min at 15°C–22°C.

Component	Volume
BbsI digested plasmid from step 5 (50 ng)	X μl
Phosphorylated and annealed oligo duplex (diluted 1:200) from step 6c	1 μL
5× T4 DNA Ligase Buffer	2.5 μL
ddH20	up to 10 μL
Subtotal	**10 μL**
T4 ligase (1 unit)	1 μL
Total	**11 μL**

e. After the ligation step, each gRNA should be incorporated into the gRNA scaffold position (Figure 4). Validate the cloning results by Sanger sequencing using the U6 primer.

⚠ CRITICAL: It is recommended that the ligation reactions be directly used for bacterial transformation to increase transformation efficiency, alternatively they could be stored at 4°C until the following day.

Bacterial transformation and plasmid DNA isolation

Timing: 3 days

7. To transform bacterial strains and yield the maximum amount of plasmid DNA:
   a. Add half of the ligation reaction (5.5 μL) from step 6d directly into a tube containing ~50 μL of DH5a competent cells (#18258012) thawed on ice, tap gently and incubate for 30 min on ice.
   b. Heat-shock the competent cells at 42°C for 30 s.
   c. Incubate on ice for 10 min.
   d. Add 1 mL LB medium and incubate at 37°C on a shaking platform at 140 rpm for 45 min.
   e. Centrifuge the tube at 1,500 × *g* for 5 min. Discard the supernatant but keep 100 μL to resuspend the bacterial pellet.
   f. Plate the mixture on a 100 mm LB agar plate containing 100 μg/mL ampicillin and incubate the plate upside down for 12–15 h at 37°C.

g. Using a pipette tip, transfer single colonies from the plate in separate tubes, each one containing 3 mL LB broth and 100 μg/mL ampicillin.
h. Incubate for ~6 h at 37°C, shaking at 140 rpm.
i. In a flask (#4980-300) containing 200 mL LB broth with 100 μg/mL ampicillin add 200 μL from the liquid bacterial culture and incubate for 12–15 h at 37°C, shaking at 140 rpm.
j. Pellet the culture by centrifugation for 10 min at 2,800 × *g* rpm at 4°C.
k. Remove the supernatant and store the pellet at −20°C for processing later, or directly proceed to plasmid extraction using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fpurelink_hipure_plasmid_filter_purification_man.pdf).
l. Measure the concentration of each purified plasmid using a NanoDrop spectrophotometer (Q3000).

Note: In the case that bacterial transformation of the cut vector alone yields multiple colonies, Calf intestinal alkaline phosphatase (CIP) (#M0290) could be added to the digested plasmids to prevent self-ligation of linearized cloning vectors.

Cell culture of HBECs before transfection

Timing: 10–15 days

8. Human Bronchial Epithelial Cells (HBECs) are cultured based on the protocol by Komseli et al. (Komseli et al., 2018). Specifically:
   a. Thaw one HBEC cryovial containing ~2 × 10^6 cells and transfer the content in a 15 mL Falcon tube containing 5 mL of Keratinocyte Serum-Free Medium supplemented with 50 μg/mL Bovine Pituitary Extract and 5 ng/mL human epidermal growth factor (EGF).
   b. Spin down at 500 × *g* at 15°C–22°C, remove completely the supernatant and resuspend the cell pellet in 10 mL Keratinocyte Serum-Free Medium (without antibiotics). The content is transferred in a 100 mm culture dish suitable for adherent cell culture (#664160).

⚠ CRITICAL: HBECs must be cultured in serum-free medium, as serum exerts toxic effects severely affecting their proliferation and survival.

Note: Avoid using antibiotics in the Keratinocyte medium, as they may induce stress.

   c. Replenish the culture medium every other day.
9. When the cells are confluent:
   a. Remove Keratinocyte medium and wash once with 1× PBS.
   b. Trypsinize cells in 1 mL trypsin/EDTA (#15400054) and incubate at 37°C for 5 min.
   c. Add an equal (or larger) amount of trypsin neutralizer (#R002100) and split cells by transferring 1/3 or 1/2 of the final volume in a 15 mL tube.

⚠ CRITICAL: Trypsin neutralizer is essential for inactivating trypsin, as serum is not contained in the medium.

   d. Pellet down the cells by centrifugation at 500 × *g* for 5 min at 15°C–22°C. Remove all the supernatant.
   e. Resuspend the pellet in Keratinocyte medium and subsequently seed cells in 100 mm cell culture plates.

⚠ CRITICAL: Do not exceed a 1:3 dilution when splitting, as cell viability may dramatically decrease.

f. Repeat steps 9a–9e every time cells become confluent to maintain a growing HBEC culture.

⚠ CRITICAL: HBECs should be cultured for at least 10 days after thawing to achieve good transfection efficiency.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
MAX Efficiency™ DH5α Competent Cells	Invitrogen	Cat# 18258012
Chemicals, peptides, and recombinant proteins		
BbsI	New England Biolabs (NEB)	Cat# R0539S
NEBuffer™ r2.1	New England Biolabs (NEB)	Supplied with BbsI
Agarose	Sigma-Aldrich	Cat# A4018
SYBR Safe DNA gel stain	Invitrogen	Cat# S33102
DNA ladder 100 bp	New England Biolabs (NEB)	Cat# N3231S
10× T4 DNA Ligase Reaction Buffer	New England Biolabs (NEB)	Cat# B0202S
T4 PNK	New England Biolabs (NEB)	Cat# M0201S
5× T4 DNA Ligase Buffer	Invitrogen	Cat# 46300018
T4 DNA ligase	Invitrogen	Cat# 15224017
LB broth	CONDA	Cat# 777491
Agar	PanReac-AppliChem	Cat# A0949
Ampicillin	PanReac-Applichem	Cat# A0839
Keratinocyte Serum-Free Medium	Invitrogen	Cat# 17005-075
PBS 1×	Biowest	Cat# L0615-500
0.5% Trypsin/EDTA 10×	Thermo Fisher Scientific	Cat# 15400054
Trypsin neutralizer	Thermo Fisher Scientific	Cat# R002100
KCl	PanReac-Applichem	Cat# 131494
TRIS	PanReac-Applichem	Cat# A1086
MgCl2	Apollo Scientific	Cat# IN1017
IGEPAL CA-630	Sigma-Aldrich	Cat# I3021
Tween 20	Sigma-Aldrich	Cat# P1379
Proteinase K	Invitrogen	Cat# AM2546
FuGENE ® HD Transfection Reagent	Promega	Cat# E2311
Opti-MEM	Thermo Fisher Scientific	Cat# 11058021
Pen/Strep	PAN-Biotech	Cat# P06-07100
10× Dream Taq Buffer	Thermo Fisher Scientific	Cat# B71
dNTPs	Thermo Fisher Scientific	Cat# R0181
Dream Taq Polymerase	Thermo Fisher Scientific	Cat# EP0702
Calf intestinal alkaline phosphatase (CIP)	New England Biolabs (NEB)	Cat# M0290
Glacial acetic acid	PanReac-AppliChem	Cat# 131008
EDTA	CALBIOCHEM-NOVAGEN	Cat# 4005-OP
DAPI	Thermo Fisher Scientific	Cat# 62247
Critical commercial assays		
Zymoclean Gel DNA Recovery Kit	Zymo research	Cat# D4001
PureLink™ HiPure Plasmid Filter Maxiprep Kit	Invitrogen	Cat# K210017
QIAquick PCR Purification Kit	QIAGEN	Cat# 28104
Experimental models: Cell lines		
HBEC-CDC6 Tet-ON	Komseli et al. (2018)	Species: Human; Cell line sex: female. Parental cells are known as HBEC-3KT.

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
gRNA1 Fw		CACCGCACCATGGAAACTGAGATA
gRNA1 Rev		AAACTATCTCAGTTTCCATGGTGC
gRNA2 Fw		CACCGAGCACACAAATGCTCAAAGC
gRNA2 Rev		AAACGCTTTGAGCATTTGTGTGCTC
hU6 Primer		GGGCCTATTTCCCATGATTCCT
Primer F1		GGCTCCCGTGCTCAAAGTAT
Primer R1		TGTTAGCATGATGTGCCCCT
Primer F2		TTGAGGAGCCAGAGGCAAAG
Primer R2		AGATCATACTTCCCCCACTCCT
Primer F3		AAGCTCTGAAGCCAATCCCC
Primer R3		TTGGTTTGGTTCCCTGCACA
Recombinant DNA		
pSpCas9(BB)-2A-GFP (PX458)	Ran et al. (2013)	pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid cat# 48138 ; http://n2t.net/addgene:48138; RRID:Addgene_48138)
pU6-(BbsI)_CBh-Cas9-T2A-mCherry	Chu et al. (2015)	pU6-(BbsI)_CBh-Cas9-T2A-mCherry was a gift from Ralf Kuehn (Addgene plasmid cat# 64324; http://n2t.net/addgene:64324; RRID:Addgene_64324)
Software and algorithms		
CRISPOR	http://crispor.tefor.net/	CRISPOR, RRID:SCR_015935
Primer-BLAST	https://www.ncbi.nlm.nih.gov/tools/primer-blast/	Primer-BLAST, RRID:SCR_003095
UCSC Genome Browser	https://genome.ucsc.edu/	UCSC Genome Browser, RRID:SCR_005780
Other		
100 mm culture dish	Greiner Bio-One	Cat# 664160
Neubauer glass chamber	Marienfeld Superior	Cat# 0640010
6-well plate	Greiner Bio-One	Cat# 657160
96-well plates	Greiner Bio-One	Cat# 650180
24-well plates	Greiner Bio-One	Cat# 662160
12-well plates	Greiner Bio-One	Cat# 665180
300 mL flasks	Life sciences	Cat# 4980-300
Q3000 UV Spectrophotometer	Quawell	N/A
FACS Aria III	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

The Quick Lysis Buffer recipe was used for genomic DNA preparation in step 5 of the "screening for inverted clone validation" section.

Quick lysis buffer recipe (for quick genomic DNA preparation)		
Reagent	Final concentration	Amount
500 mM KCl	50 mM	5 mL
500 mM TRIS pH: 8.3	10 mM	1 mL
5 mM $MgCl_2$	2.5 mM	25 mL
NP40	0.45%	225 µL
Tween 20	0.45%	225 µL
ddH_2O		18.5 mL
Total		**50 mL**
20 mg/mL Proteinase K	0.4 µg/µL	1 µL per 50 µL Lysis Buffer

Quick lysis buffer can be stored at 4°C for up to 3 weeks without Proteinase K, which should be added on the day of the cell lysis.

10× TAE (for agarose gel preparation)		
Component	Final concentration	Amount
Tris	0.4 M	48.4 g
Glacial acetic acid	1.14%	11.4 mL
EDTA	12 mM	3.7 g
dH20		up to 1 L dH2O
Total		**1 L**

10× TAE buffer can be stored at 15°C–22°C for several months. 10× TAE is diluted to 1× working solution.

Agarose gel recipes		
Component	1.8% agarose gel	1.3% agarose gel
Agarose	0.8 g	1.3 g
1× TAE	up to 100 mL	up to 100 mL
Total	**100 mL**	**100 mL**
SYBR Safe DNA gel stain	10 µL	10 µL

Agarose gels should ideally be used immediately after preparation or stored in the dark at 4°C, in 1× TAE buffer for up to 24 h following preparation.

STEP-BY-STEP METHOD DETAILS

Seeding and transfection of HBECs

Timing: 3 days

1. Seeding of HBECs prior to transfection:
   a. Harvest HBECs as described in steps 9 (a–e) of the "cell culture of HBECs before transfection" section above.
   b. Count cells using a Neubauer glass chamber.
   c. Seed ~8 × 10^4 cells in each well of a 6-well plate containing 2 mL keratinocyte medium to subsequently transfect the cells with the gRNA/Cas9 expressing vectors.
2. Two days after plating, the keratinocyte medium is replenished and double transfection with the gRNA/Cas9 expressing vectors can be performed. HBECs are transfected with 1.5–2.5 µg of each plasmid DNA using the FuGENE ® HD Transfection Reagent (#E2311).
   a. Prepare the Transfection reagent: DNA mixture in a tube using a 4:1 FuGENE® HD Transfection Reagent: DNA ratio, and add the mixture in 200 µL Opti-MEM (#11058021). For example, 3 µg of total plasmid DNA mixed with 12 µL FuGENE are added in 200 µL Opti-MEM.
   b. Resuspend by thorough pipetting and incubate for 15 min at 15°C–22°C.
   c. Add the mixture dropwise in each transfection well containing 2 mL keratinocyte medium, in antibiotic- and serum-free conditions.

⚠ CRITICAL: Avoid using antibiotics in the transfection medium, as they may reduce the HBEC transfection efficiency due to increased cellular stress.

Note: Use only one gRNA per breakpoint per transfection.

Note: Other adherent cell lines which are normally cultured in the presence of serum (e.g., Li-Fraumeni) can easily be transfected using serum-containing medium, as serum does not interfere with the FuGENE reagent.

   d. After 24 h, HBECs can be harvested to assess plasmid expression and/or used in downstream analyses.

⚠ CRITICAL: To minimize stress and achieve high co-transfection efficiency up to 35%, transfect HBECs (or other challenging cell lines) at a confluency of ~80% (i.e., two days after seeding cells at the density indicated in step 1c.

Single-cell sorting by fluorescence-activated cell sorting (FACS)

Timing: 1 day

3. Following transfection, HBECs are subjected to FACS sorting:
   a. 24 h after transfection, trypsinize and collect HBECs as described in steps 9a–9d of the "cell culture of HBECs before transfection" section.
   b. Wash once with 1× PBS and spin down at 500 × *g* for 5 min at 15°C–22°C.
   c. After discarding the supernatant, resuspend the cell pellet in 500 μL Opti-MEM or Keratinocyte medium and keep on ice until sorting.

Note: Cellular viability can be estimated by adding 4′,6-Diamidino-2-Phenylindole (DAPI) (at a final concentration of 500–1,000 ng/mL) into a portion of the transfected cells resuspended in 500 μL 1× PBS, directly before sorting. Only the DNA of non-viable cells is stained by DAPI due to the inability of DAPI to pass through intact cell membranes of living cells. In the case of HBECs the percentage of dead cells was estimated at 4%–7% of the population.

   d. Perform single-cell sorting of the cell suspension using a flow cytometer (BD FACS Aria III). Adjust the settings such that only double-positive single cells expressing both GFP and mCherry (GFP+/mCherry+) will be sorted.
   e. Prepare 96-well plates (#650180) for cell sorting, by adding in each well 100 μL keratinocyte medium supplemented with 1% Pen/Strep to avoid contamination during FACS. Prepare at least 4 × 96-well plates.
   f. Sort cells by plating one cell per well of a 96-well plate.

⚠ CRITICAL: Given that the percentage of single cells surviving after single cell sorting is very low (approximately 30%), it is crucial to fill as many 96-well plates as possible, as this will facilitate the screening procedure and minimize experimental risk.

Propagation of clones

Timing: 3–4 weeks

4. HBECs that survived after seeding into 96-well plates as single cells will gradually start proliferating, giving rise to separate clones in each well.
   a. Allow single cell cultures to recover by incubating undisturbed in humid 37°C/5% CO_2 chamber for 7 days after FACS.
   b. Add 100 μL antibiotic-free keratinocyte medium and start monitoring the clones under the microscope.
   c. Replenish the medium every week: Remove carefully the medium from each well using a 100 μL pipette or a multi-channel pipette, trying not to touch the bottom and slowly add 100 μL fresh Keratinocyte medium.
   d. Once single cell-derived clones reach full confluency, the screening procedure can commence.

Note: The use of a multi-channel pipette is highly recommended for easier and faster handling of the clones.

Screening for inverted clone validation

Timing: 1–2 weeks

DNA is first extracted from each clone separately and the presence of the inversion is queried via PCR.

5. For quick preparation of genomic DNA (Charette and Cosson, 2004):
   a. After aspirating the medium and washing once with 1× PBS, harvest the cells derived from each well of the 96-well plates by adding 30 µL Trypsin/EDTA 1× and incubating for 5 min at 37°C.
   b. Add an equal or larger volume of Trypsin Neutralizer Solution and resuspend thoroughly by pipetting.
   c. For each clonal culture, transfer half of the amount of solution containing the trypsinized cells in a new well of a 96-well plate containing 200 µL (or more) of keratinocyte medium.

⚠ CRITICAL: For HBECs and other sensitive cell lines which normally require centrifugation for total trypsin removal, the addition of a larger amount of medium (≥ 200 µl) in the new 96-well plates will yield equivalent results in regards to propagation.

   d. Lyse the cells which were not transferred into new 96-well plates by directly adding 30 µL of Quick Lysis Buffer (see materials and equipment section above) and transfer the lysate in an Eppendorf tube.
   e. Heat for 45 min at 60°C, followed by 10 min at 80°C for Proteinase K inactivation.
   f. The lysate contains the genomic DNA, and 4 µL of the lysate can be used directly as template for PCR.
   g. The rest of the lysate can be stored at −20°C for 1–2 weeks or −80°C for long-term storage.
6. For the screening process:
   a. Set up the PCR reactions using F1/F2 and R1/R2 primers (each pair in a separate reaction) to verify the presence of the inversion and exclude "non-inverted" clones. Set up the reaction for each clone as described below:

Component	Volume
DNA template	4 µL
10× Dream Taq Buffer	2.5 µL
10 mM dNTPs	0.5 µL
10 µM primer mix	2.5 µL
Dream Taq Polymerase (1.25 units)	0.25 µL
ddH20	up to 25 µL
Total	**25 µL**

⚠ CRITICAL: Before screening, it is essential that PCR conditions are optimized for the set of primers used. It is recommended to first use the F1/R1 and F2/R2 primer combinations on wild-type genomic DNA template, which should result in an unambiguous PCR product corresponding to the wild-type alleles. For guidance regarding the design of primers for efficient targeting, please refer to the "design of primers for identifying the inversion" section above.

   b. Run the reactions in a 1.3% agarose gel (for recipe see Table in the "materials and equipment" section prepared with SYBR Safe DNA gel stain (#S33102). The clones yielding a PCR product (at ~770 bp and ~1,300 bp for F1/F2 and R1/R2, respectively, in our experimental setup) harbor at least one inverted allele (Figure 5A).

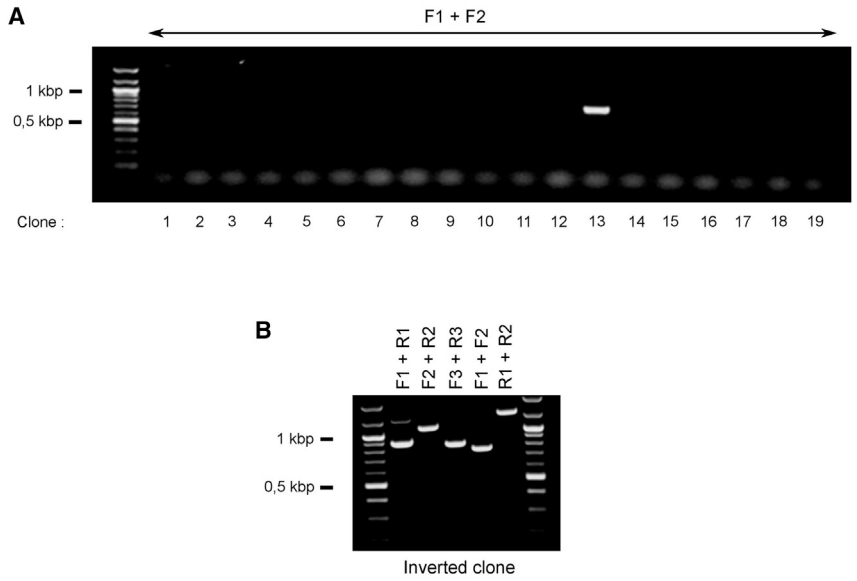

Figure 5. Validation of the engineered inversion through PCR

(A and B) Agarose gel images representing: (A) PCR screening of CRISPR-Cas9-engineered clones using F1+F2 primers. Clone 13 possibly harbors the desired inversion. (B) Identification of heterozygosity regarding the inversion; F1+R1: amplification of wild-type product at 921 bp, F2+R2: amplification of wild-type product at 1,100 bp, F3+R3: amplification of the *BHLHE40* gene located between the breakpoint sites at 856 bp, F1+F2: amplification of inverted breakpoint 1 region at ~770 bp, R1+R2: amplification of inverted breakpoint 2 region at ~1,300 bp. The presence of bands for both wild-type and inverted regions in the same clones indicates heterozygosity.

c. To assess heterozygosity, run PCR reactions as described in step 6a on the same clones, this time using the F1/R1 and F2/R2 primer combinations (again, each primer combination in a separate reaction). Amplification with these sets of primers will reveal the presence of a wild-type allele in addition to the inverted one previously identified, which indicates heterozygosity (Figure 5B). Absence of amplification using the F1/R1 and F2/R2 primer combinations indicates homozygosity regarding the inversion.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	1 min	1
Denaturation	95°C	30 s	30 cycles
Annealing	63°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	15 min	1
Hold	4°C	Forever	

d. To conclusively validate the engineering of the desired inverted area, PCR products should be first purified using the QIAquick PCR Purification Kit (#28104, https://www.qiagen.com/us/resources/download.aspx?id=95f10677-aa29-453d-a222-0e19f01ebe17&lang=en) and submitted for Sanger sequencing using the F1 or F2 primer for the inverted breakpoint 1 and R1 or R2 for the inverted breakpoint 2.

Note: The inversion occurs at low frequency after CRISPR-Cas9 editing (<1.5% in HBECs), therefore a large number of clones should be screened via PCR to identify the few ones harboring the inversion.

Propagation of the inverted clones

Timing: 2–3 weeks

The clones identified as "inverted" should be gradually expanded in the case of HBECs or other sensitive cell lines. Specifically, cells should be initially transferred from each well of a 96-well plate to a well of a 24-well plate and then to a 12-well plate before the cells can be safely transferred to a 6-well plate. This is an essential step to avoid additional stress of the cells growing in the presence of Trypsin/EDTA and Neutralizer (see step 5c in the "screening for inverted clone validation" section), which cannot be directly removed, as centrifugation of such a small pellet is not recommended at this critical step, to prevent cell loss.

⚠ CRITICAL: HBECs should be transferred to larger wells only upon reaching confluency, as sub-confluent HBEC cultures exhibit lower viability and propagate at a slower rate.

EXPECTED OUTCOMES

Estimated yield of plasmid concentration on the NanoDrop spectrophotometer after bacterial transformation and use of the PureLink™ HiPure Plasmid Filter Maxiprep Kit (see Section "bacterial transformation and plasmid DNA isolation"): 0.5–2 μg/μL. Estimated percentage of GFP+/mCherry+ HBEC single cells surviving following FACS sorting: 30%. Estimated DNA yield following PCR product purification using the QIAquick PCR Purification Kit (see Section "screening for inverted clone validation", step 6d): 50–150 ng/μL. Estimated inversion efficiency (clones containing at least one "inverted" allele) in the surviving single-cell deriving HBEC cultures: <1.5%.

LIMITATIONS

One possible limitation of the described protocol stems from the fact that "inverted" clones derive from single cells sorted through FACS. As HBECs are non-cancerous cells whose survival may be compromised in sub-confluent culture conditions, it is likely that a proportion of cells harboring inversions fail to propagate beyond the single cell stage. This will likely have an impact on the yield of "inverted" clones during the screening process. Expanding the protocol to cell lines demanding a higher level of confluency than HBECs to maintain survival may, therefore, require further optimization.

TROUBLESHOOTING

Problem 1

Single cells sorted by FACS display compromised survival. (step-by-step method details, step 3).

Potential solution

Single-cell isolation using FACS is highly recommended, because it is a relatively straightforward method to acquire clonal cultures without the addition of selection chemicals which would potentially confer toxic effects. Single cell survival may partially improve by adjusting various parameters related to the FACS sorting process *per se*, such as using fresh buffer solutions, adjusting the nozzle size depending on the cell line and maintaining cells in the required temperature until the time of sorting. Those actions will contribute towards minimizing the stress possibly induced by external factors.

If optimizing FACS conditions is not sufficient to improve single cell survival, a more laborious approach may be implemented, which involves sorting clumps of cells rather than single cells and subsequently performing serial dilutions in order to achieve single cell cultures at a later stage. This, of course, may lead to a significant workload increase as the risk of cell clumps not consisting exclusively of GFP+/mCherry+ double positive cells is higher.

In case FACS sorting cannot be implemented for non-HBEC cell lines, manual serial dilution could be applied to obtain single cells, however this method cannot exclude negative or single-positive cells from being propagated.

Antibiotic selection may be a potential alternative route to FACS sorting of non-HBEC cell lines, provided that the plasmid vectors carry antibiotic resistance cassettes (preferably different from one another, to facilitate the selection process) and the cell line of choice can tolerate antibiotic treatment. This method may be more time-efficient compared to single cell FACS sorting.

Problem 2

The inverted amplicon is not detectable. (step-by-step method details, step 6).

Potential solution

In the case that the primers (F1/R1 and F2/R2) cannot amplify the wild-type genomic region, make sure that the PCR conditions, and especially the annealing temperature (Tm), have been properly optimized based on the Tm of the individual primers (see "design of primers for identifying the inversion" section). If primer design parameters seem to be met but there is still no PCR product, we recommend setting up the PCR reactions trying other sets of primers.

In the case that the F1/R1 and F2/R2 primers are able to amplify the wild-type genomic regions, but their combinations (F1/F2 and R1/R2) fail to yield any PCR product corresponding to the inverted regions, this is an indication that the gRNAs used may be inefficient and we recommend using the next best gRNA candidate sequence based on the CRISPOR criteria (see "design of gRNAs" section, step 2).

Problem 3

The inverted breakpoints have been identified by PCR, but after clonal propagation the amplicons are gradually withdrawn (step-by-step method details, "propagation of the inverted clones" section).

Potential solution

This could potentially be attributed to the presence of more than one cells plated in individual wells of the 96-well plates during FACS sorting. FACS generally ensures that single cells are sorted; however, a doublet may be occasionally plated instead of a single cell. Sorting cells in as many as possible 96-well plates offers the possibility to reduce such contaminations.

Another possibility is related to cross-contamination during cell culturing, which may occur when handling wild-type and "inverted" clones at the same time. This can be largely prevented with the systematic use of filter pipette tips, to prevent unwanted transfer of cells among different cultures.

Problem 4

Only one of the inversion breakpoint regions (F1/F2 or R1/R2) is amplified by PCR (step-by-step method details, step 6).

Potential solution

This may possibly occur as a result of an extensive break, preventing the binding of primers to a region proximal to the breakpoint. A possible solution is to design a new set of primers binding further away from the breakpoint (see "Design of primers for identifying the inversion" section).

Problem 5

Lipofectamine-based transfection approaches may lead to insufficient transfection efficiency or be highly toxic for non-transformed cells (step-by-step method details, step 2).

Potential solution

To minimize this problem, we used the FuGene transfection reagent (#E2311), which is a non-liposomal formulation yielding higher efficiency and reduced toxicity compared to other lipofectamine reagents which were also tried on HBECs. Another potential solution to the problem would be the

implementation of virus-based approaches, including lenti or adenoviral vectors. Those virus-based approaches would likely increase transfection efficiency, enabling replication of results and validation of a chromosomal inversion.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Professor Vassilis G Gorgoulis (vgorg@med.uoa.gr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets and code generated or analyzed during this study.

ACKNOWLEDGMENTS

We would like to thank Dr. Panagiota Kafasla and Dr. Paschalis Sideras for technical support. V.G.G. is financially supported by the European Union Horizon 2020 Research and Innovation Program under Marie Sklodowska-Curie grant agreement 722729 (SYNTRAIN). V.G.G. is also supported by the National Public Investment Program of the Ministry of Development and Investment/General Secretariat for Research and Technology in the framework of the Flagship Initiative to address SARS-CoV-2 (2020SE01300001); the Welfare Foundation for Social & Cultural Sciences (KIKPE), Athens, Greece; an H. Pappas donation; grants 775 (Hippo) and 3782 (PACOREL) from the Hellenic Foundation for Research and Innovation (HFRI); and NKUA-SARG grant 70/3/8916. A.A. is co-funded by the Hellenic Foundation for Research and Innovation (HFRI) under the 3rd Call for HFRI PhD Fellowships (fellowship number: 5747). A. Papaspyropoulos is co-funded by the Foundation for Education and European Culture (IPEP). A. Papantonis is supported by the Deutsche Forschungsgemeinschaft via the Clinical Research Unit KFO5002 project grant (CO 1568/2-1) and the Priority Program SPP2202 (grant PA 2456/11-1).

AUTHOR CONTRIBUTIONS

A.A. and A. Papaspyropoulos contributed equally to this work. A.A. and A. Papaspyropoulos contributed to writing the protocol. A. Papantonis and V.G.G. contributed to writing the protocol and supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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