Report on ARPE-19 Knockout strains using CRISPR/Cas9

1. Protocol

1.1 sgRNA design

TrueDesign™ Genome Editor online tool from ThermoFisher Scientific ™ was used for the selection of the best fitted single guide RNA (sgRNA) to target the *NFE2L2* gene. This tool allowed the sgRNA selection based on the score % and on the off-targets that better allowed the creation of a frameshift that culminated on the knockout of the human *NFE2L2* (variant 1). The selected sgRNA (5' GCGACGGAAAGAGTATGAGC 3') presented a 100 % score, no off-targets and belonged to the initial segments of the gene sequence (exon 2).

1.2 CRISPR-Cas9 system delivery by electroporation

Generation of gene of interest (*NFE2L2*) knockouts in ARPE-19 cells was preformed using the Alt-R CRISPR-Cas9 system (Integrated DNA Technologies, IDT) and the Neon Transfection System Kit (Invitrogen, 10 µL, MPK1025K). Low passage, ARPE-19 cells were grown to 70 % confluence in a 25 cm^2 t-flask. Cells were washed with 3 mL of PBS 1x, followed by the addition of 2.5 mL of TripLE, and incubation for 5 min at 37°C and 5 % CO_2. After addition of 3 mL of supplemented DMEM, cells were resuspended and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in 1 mL of PBS 1x, and cells were counted in a Countess Cell Counting Chamber Slides (Invitrogen, C10283). Cells were pelleted again and re-suspended in 9 µL of Resuspension Buffer (Buffer R, Neon Transfection 10 µL kit, Invitrogen, MPK1025K) to a final concentration of 5.6 x 10^{-5} cells/mL. The designed sgRNA, HS. Cas9. NFE2L2.1. AB (IDT, 234783445) was diluted to a final concentration of 44 µM with the assistance of IDT resuspension calculator. Then, the Ribonucleoprotein (RNP) complex was formed by diluting 0.7 µL of Alt-R S.p. Hifi Cas9 Nuclease V3 (IDT, 1081060) in 0.5 µL of Buffer R for a final concentration of 36 µM, to then mix the diluted Alt Cas9 enzyme with 1.2 µL of sgRNA. The complexation reaction was left to progress at room temperature for 15 minutes. Meanwhile, the 100 µM Alt-R Cas9 Electroporation enhancer (IDT, 1075916) was diluted in molecular water to reach a final concentration of 10.8 µM (working solution). Finally, 1 µL of the RNP complex was mixed with 9 µL of cell suspension and 2 µL of Alt-R enhancer working solution. Electroporation was then carried out with an intensity of 1350 mV, along 2 pulses of 20 ms, accordingly to manufacturer's instructions. Cells were seeded in a 12-well plate with 2 mL of pre-warmed supplemented DMEM without antibiotics. Cells were left to grow until confluency was reached.

1.3 Super-low confluency and clone selection

When confluency was reached, cells were passed to a 6-well plate at six different dilutions (1:25, 1:50, 1:100, 1:200, 1:500 and 1:1000). As soon as cells started to form aggregates, different clones were isolated from the different dilution wells with a 200 µL tip into a 48-well plate. Once clones started to reach confluency, cells were expanded into 12-well plates either for immunoblotting, genomic DNA extraction or freezing.

1.4 Genomic DNA isolation

To confirm if the gene editing process was successful in the *NFE2L2* knockout, approximately 100 000 cells per well were seeded on a 24-well plate and incubated to reach confluence. Genomic DNA (gDNA) was isolated from the samples using an NZY Tissue gDNA Isolation Kit (50) (NZYTech genes & enzymes, MB13502) according to the manufacturer's instructions. After purification, the DNA concentration was measured at NanoDrop™ 2000 (ThermoFisher Scientific).

2.2.5 Primer design

Primer-BLAST online tool from the National Centre of Biotechnology Information (NCBI) was used for the selection of the most fitted primers to amplify the gene edited region of the *NFE2L2*. In this tool it was inserted the previous and following gDNA sequence region of the *NFE2L2* for which the sgRNA was designed. Additionally, all settings were set as default with exception for the PCR product minimum size that was set for 200 base pairs (bp). The selected primers are presented in Table 1.

Table 1. Primers sequences used in PCR

Gene	Sequence 5' to 3'		Product Length (bp)
NRF2Seq.1	FW	TTCCCACCATCAACAGTGGC	507 bp
	RV	GGAGGCTGAGGTTGGAAAGTA	
NRF2Seq.2	FW	CCCACCATCAACAGTGGCATA	409 bp
	RV	ACCTGCCATAACTTTCCCAAG	

1.6 Polymerase chain reaction, electrophoresis, and sequencing

Amplification was achieved using the DreamTag™ Green PCR Master Mix 2x (ThermoFisher Scientific, K1081), 10 µM of the designed forward and reverse primers listed in Table 2 and 200 ng of gDNA that was used as a template for the polymerase chain reaction (qPCR). qPCR was performed on a PCR Biorad MyCycler (BIO RAD, 170-

9703) and the amplification products were confirmed by agarose gel electrophoresis. The agarose solution was prepared to reach the final concentration of 1.5 % w/v agarose (Lonza, 50004)/Tris-acetate-EDTA (TAE) buffer (Bio-Rad, 161-07773). The solution was heated until agarose was dissolved and supplemented with SYBER Safe DNA Gel Stain 10 000x (Invitrogen, S33102) in a 1:10 000 dilution. The gel was loaded with 3 µL of O'Gene Ruler 1Kb DNA Ladder (ThermoFisher Scientific, SM1163) and 10 µL of sample. Electrophoresis was revealed under UV light using a ChemiDoc imaging system by Bio-Rad. PCR products were then purified using the QIAquick® PCR Purification Kit (Qiagen, 28104) following the manufacture instructions and DNA quantified using NanoDrop™ 2000 (ThermoFisher Scientific). Finally, separate samples were prepared with 10 µL of sample and 3 uL of forward primer (Table 1) and sent for sanger sequencing by StabVida™.

2. Results

The CRISPR/cas9 gene editing technique was used to target *NFE2L2* exon 2 and the consequent generation of an RPE-NRF2-knockout strain (Figure 1 A).

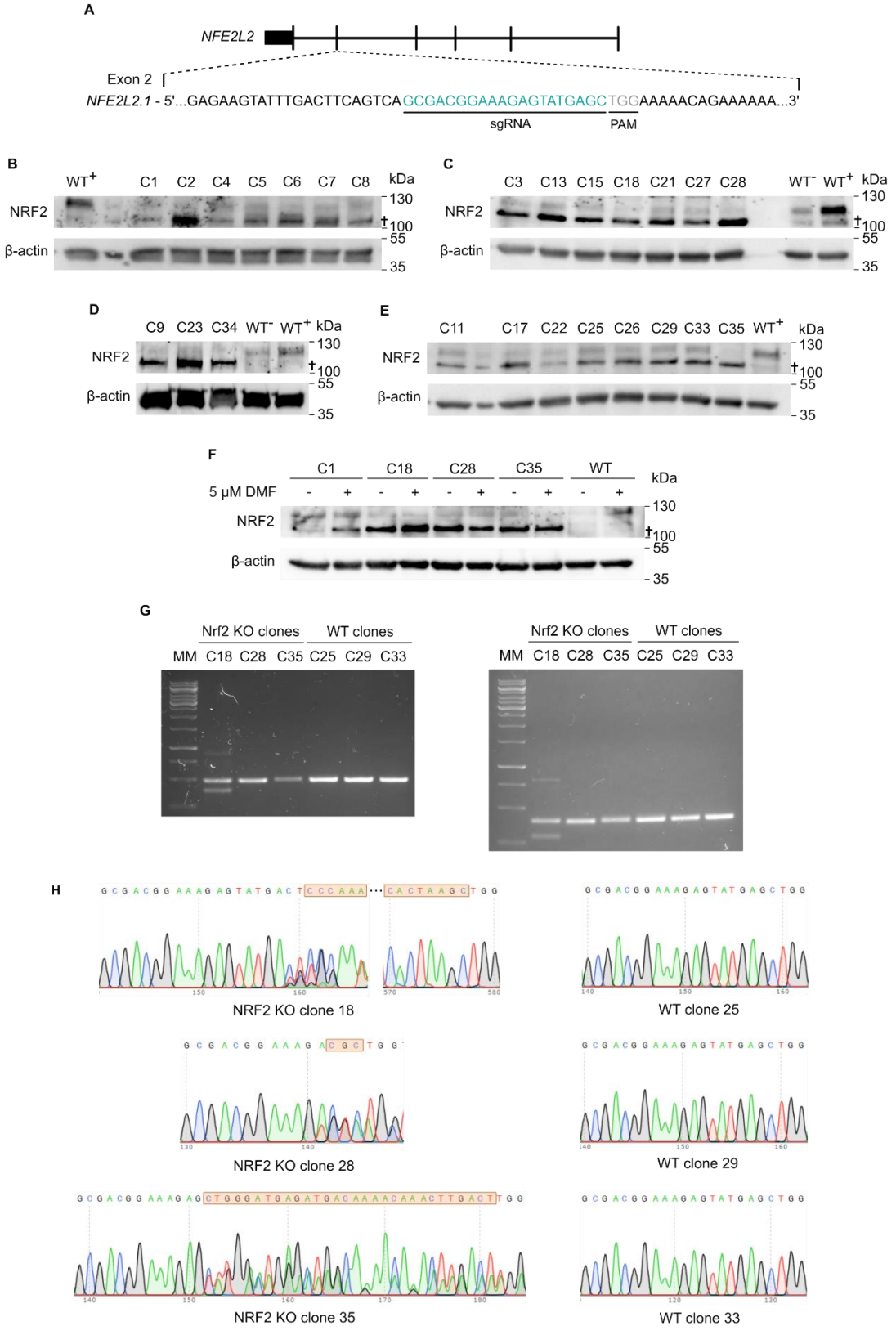

Figure 1. An NRF2 knockout (KO) in RPE human cells was successfully generated and confirmed by sequencing. (A) CRISPR/Cas9 gene editing was used to generate ARPE-19 NRF2 KO strains by targeting the *NFE2L2* exon 2. sgRNA and PAM sequences are represented in green and grey, respectively. From this procedure, twenty-five clones were isolated and grown for analysis. **(B-E)** Immunoblotting analysis of NRF2 protein levels of the NRF2 KO clones. For further analysis, three positive (clones 18, 28 and 35) and three

negative (clones 25, 29 and 33) KO clones were selected. **(F)** Immunoblotting analysis of NRF2 protein levels of selected NRF2 KO clones incubated with 5 μM DMF for 4 hours. Protein levels were normalized to β-actin and is representative of N=1 independent experiments. **(G)** Confirmation of the amplification of the selected clones for sequencing. For this step, two sets of primers were designed. Results for the first forward and reverse primers are on the left and the second forward and reverse primers on the right. **(H)** Schematic representation of sanger sequencing of selected KO and WT/control clones. The frameshift mutations obtained for each KO strain are represented by an orange box. WT $^{-}$, Wild-type cells; WT^{+}, Wild-type cells incubated with 5 μM DMF. †NRF2 isoform.

From this procedure, twenty-five clones were isolated and grown, and the efficacy of the process was assessed by immunoblotting with WT RPE cells with and without 5 μM DMF incubation (Figure 1 B-E). Results show some promising KO candidates such as clones 1, 18, 28 and 35 which reveal the disappearance of the NRF2 band below the 130 kDa.

To further confirm these results the proposed clones were incubated with 5 μM DMF for 4 hours (Figure 1 F). This experiment retrieved no differences in NRF2 protein levels between treated and non-treated clones. Therefore, the targeted region of three positive (clones 18, 28 and 35) and three negative clones (25, 29 and 33) was amplified by PCR with two specifically designed primers and amplification was confirmed by agarose gel electrophoresis (Figure 1 G). Clone 18, contrarily to the remaining, ones revealed an amplification of three different fragments for both designed primers. This behaviour is typical of mixed deletion, high deletion steps or end-joining which can be favourable for the fulfilment of our aim. As all clones were successfully amplified, the positive and negative clones were sent for sequencing. Sequencing results of all negative clones showed no changes when compared to the WT sequence, confirming that the technique was not successful in creating the NRF2 KO (Figure 1 H). Meanwhile, positive clones showed endogenous corrections of the nicks induced by CRISPR/Cas9 which created a frameshift, and consequently the KO of NRF2 (except for one isoform migrating above 100 kDa). Particularly, clone 18 revealed a 418 bp repair, confirming that the three previously found PCR products were in fact caused by this extensive insertion (Figure 1 H).