Supporting Information for

Complete POR ablation in human adrenal cells reveals steroidogenic rerouting and variant-dependent loss of cytochrome P450 support

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Supporting Information Text

Supplementary Methods

Cell lines and culture conditions

HEK293T, NCI-H295R and their POR knockout derivate cell lines were cultured at 37°C in a humidified incubator with 5% CO_2 under sterile conditions. Media were refreshed every 2–3 days, and cells were routinely examined for morphology and contamination. Cells were maintained in exponential growth phase and were passaged at 70–80% confluence following PBS wash and enzymatic detachment with trypsin–EDTA. After detachment, cells were resuspended in complete medium and seeded for further experiments. HEK293T, HEK293T POR KO, and HEK293T POR p.P228L cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin (Gibco™, 11965092, A5670901, 15070063). NCI-H295R, POR knockout clones T1-4 and T1-6 cell lines were maintained in DMEM/F12 supplemented (Gibco™, 11320033) with 2.5% Nu-Serum, 0.1% ITS (Corning®, 355500, 354350), and 1% penicillin–streptomycin. For all assays, cells were plated 24 h prior to experiment to ensure proper attachment and recovery. Only cultures with normal morphology and high viability were used for analysis.

Plasmid construction for site-directed mutagenesis

Mutant POR expression constructs were generated using the mammalian expression vector pcDNA3.1(+)_POR_WT, encoding human wild-type NADPH–cytochrome P450 oxidoreductase (POR; NM_001395413), as the template. Site-directed mutagenesis was performed to introduce the following POR variants: p.A287P, p.P228L, p.delP399_E401, and p.R457H. Mutagenesis PCR reactions were carried out using Pfu DNA Polymerase (Promega, M7741) with 1 µg of plasmid DNA according to the manufacturer's instructions. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 20 cycles of 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 8 min, with a final extension at 72 °C for 10 min. Following amplification, parental template DNA was removed by digestion with 10 U DpnI (Thermo Scientific, ER1701) for 1 h at 37 °C. The resulting reaction mixture was used to transform DH5α chemically competent Escherichia coli using standard heat-shock transformation procedures. Plasmid DNA was isolated from bacterial cultures, and the presence of the intended mutations was confirmed by Sanger sequencing. HEK293T cells were transfected with the constructed plasmids using Lipofectamine™ 2000 (Invitrogen, 11668019) according to the manufacturer's protocol. Cells were seeded one day prior to transfection at a density of 2.5×10^5 cells per well in 12-well plates. For each transfection, 1 µg plasmid DNA was mixed with 1.5 µL Lipofectamine™ 2000 in 100 µL Opti-MEM medium (Gibco, 31985070) and added to the cells. After 48 h of incubation at 37 °C in 5% CO_2, transfected cells were seeded for the following assays.

Plasmid construction for CRISPR/Cas9 mediated gene knockout

For POR knockout in HEK293T cells, three guide RNAs (gRNAs) targeting the POR gene were designed using the CRISPOR[1], Benchling, and CHOPCHOP[2] online tools. The gRNA sequences were cloned into the BbsI restriction sites of the eSpCas9-2A-GFP plasmid (PX458, GenScript) as was described[3,4]. Briefly, oligonucleotides encoding gRNAs were designed with appropriate BbsI-compatible overhangs for cloning into the eSpCas9-2A-GFP plasmid PX458. For each gRNA, complementary forward and reverse oligonucleotides were synthesized, phosphorylated, annealed, and ligated into the BbsI-digested vector backbone. First, oligonucleotides were resuspended in nuclease-free water to a concentration of 100 µM. For oligonucleotide phosphorylation and annealing, 1 µL of each oligonucleotide was mixed with H_20, T4 DNA ligase buffer and T4 polynucleotide kinase in a 10 µL reaction. The mixture was incubated at 37 °C for 60 min, followed by 95 °C for 5 min, and gradually cooled to 25 °C to allow annealing. Next, the annealed oligonucleotides were cloned into the PX458 plasmid using a restriction–ligation reaction containing BbsI (Thermo Scientific, ER1012), T4 DNA ligase and T4 DNA ligase buffer (Thermo Scientific,15224041) in a 10 µL reaction volume. The reaction was subjected to 35 cycles of digestion and ligation (37 °C for 5 min, followed by 20 °C for 5 min). The reaction mixture was used for DH5a competent cells transformation. The resulting plasmids were purified using the GenElute™ HP Plasmid Maxiprep Kit (Sigma-Aldrich, NA0310-1KT). Correct insertion of the gRNA sequences was verified by Sanger sequencing (Microsynth). HEK293T cells were transfected with the constructed plasmids using Lipofectamine™ 2000 (Invitrogen, 11668019) according to the manufacturer's protocol. For each transfection, 1 µg plasmid DNA was mixed with 1.5 µL Lipofectamine™ 2000 in 100 µL Opti-MEM medium (Gibco, 31985070) and added to the cells. After 24 h of incubation at 37 °C in 5% CO_2, transfected cells were evaluated for eGFP expression and sorted based on fluorescence using a MoFlo ASTRIOS BSL-2 cell sorter (Beckman Coulter). Single eGFP-positive cells were deposited into 96-well plates containing preconditioned culture medium and incubated at 37 °C and 5% CO_2. Colony formation was monitored, and single-cell clones were expanded. After 20 days, surviving clones were subjected to genotyping, Western blot analysis, and functional POR assays.

Editing efficiency of the designed gRNAs was evaluated by TIDE analysis[5], and the most efficient gRNA (2S_ex4POR_KO_for_eCAS9_BbsI) was selected for further experiments. This gRNA, targeting exon 4 of the POR gene, was used to generate POR knockout HEK293T cells.

POR knockout NCI-H295R cell lines (T1-4 and T1-6) were purchased from GenScript and used for subsequent experiments. During their manufacture, the gRNA – Cas9 ribonucleoprotein (RNP) complex targeting the POR gene was electroporated into NCI-H295R cells. Following electroporation, single-cell sorting was performed, and clonal cell populations were expanded.

CRISPR/Cas9 Prime Editing system construction

The following vectors were the kind gifts of Prof. Schwank and Dr. Tálas (UZH): pCMV-PEmax-tagRFP-BleoR, pEF1a-hMLH1dn and pU6-tevopreq1-GG-acceptor; the nickase vector pSpCas9n BB-2A-GFP (PX461) was purchased from GenScript.

Prime editing guide RNA (pegRNA_P228L_PBS+RTT_xPAM), including primer binding site (PBS) of 13 bp and reverse transcription template (RTT) of 19 bp[6], was designed using PRIDICT2.0[7] . It targets exon 7 in POR gene and introduces the biallelic POR mutation p.P228L (GRCh38: NC_000007.14:g.75981558C>T; NP_000932.3). In addition, a PAM-disrupting silent mutation[8] (GRCh38: NC_000007.14:g.75981562C>G; POR p.A229A) was incorporated to prevent re-editing. A nicking sgRNA (P228L_PE3_nick_S_BbsI) targeting the non-edited strand 65 bp downstream of the prime editing site was designed for the PE3 strategy[9].

The nicking sgRNA was cloned into the pSpCas9n(BB)-2A-GFP (PX461) vector, generating the PE_nick construct, as described above[3]. The pegRNA was cloned into the pU6-tevopreq1-GG-acceptor vector using a standard BbsI restriction–ligation cloning strategy. For prime editing, HEK293T cells were seeded at 6.5×10^5 cells per well in 6-well plates in 2 mL growth medium. The following day, cells were transfected using jetOPTIMUS® reagent according to the manufacturer's instructions. The transfection mixture contained 700 ng pCMV-PEmax-tagRFP-BleoR, 300 ng pegRNA plasmid, 500 ng pEF1α-hMLH1dn, and 500 ng PE_nick plasmid, combined with 2.5 µL jetOPTIMUS® reagent in 200 µL jetOPTIMUS buffer. Twenty-four hours post-transfection, the culture medium was replaced with fresh medium containing 100 µg/mL Zeocin (Thermo Scientific, J67140.XF) for selection. Selection was maintained for 10 days until non-resistant HEK293T control cells were eliminated. Surviving cells were subsequently single-cell subcloned, and monoclonal cell lines were established. The presence of biallelic POR p.P228L and POR p.A229A mutations was confirmed by Sanger sequencing.

Genotyping

To confirm CRISPR/Cas9-mediated editing of the POR gene, genomic DNA was extracted from individual clones, and the targeted region was amplified by PCR. Primers flanking exon 4 of the POR gene (POR_ex4_F and POR_ex4_R) were used to amplify the edited locus. PCR products were purified to remove residual primers and nucleotides and subjected to Sanger sequencing (Microsynth). Sequencing chromatograms were analyzed and aligned to the reference POR sequence to identify insertions, deletions, or nucleotide substitutions at the target site using SnapGene software (version 7.2.0).

Whole transcriptome sequencing

Total RNA was isolated from HEK293T, HEK293T_POR_KO, NCI-H295R, and POR knockout NCI-H295R derivatives (T1-4 and T1-6) using TRIzol™ Reagent (Invitrogen, 15596026) according to the manufacturer's instructions. RNA quality and integrity were assessed by agarose gel electrophoresis, NanoDrop spectrophotometry, and PCR (for DNAse control).

RNA sequencing was performed by Novogene Co. (UK) using the Illumina platform. mRNA was isolated from total RNA using poly-T oligo-attached magnetic beads, fragmented, and reverse transcribed to generate cDNA libraries, which were prepared by end repair, A-tailing, adapter ligation, size selection, and PCR amplification. Library quality was assessed using Qubit, qPCR, and Bioanalyzer prior to sequencing. Raw reads were filtered to remove adapters, low-quality reads, and reads containing ambiguous bases. Clean reads were aligned to the human reference genome (hg38) using HISAT2, and gene expression was quantified with featureCounts and normalized as FPKM.

Lentiviral transduction

Lentiviral particles carrying CYP17A1, CYP19A1, and CYP21A2 coding sequences were obtained from GenScript with a reported titer of 1×10^8 IFU/mL. Prior to infection, HEK293T and HEK293T_POR_KO cells were seeded in 24-well plates as 1×10^5 cells per well in 500 µL growth medium. On the following day, the medium was replaced with 300 µL fresh growth medium supplemented with polybrene (8 µg/mL). Cells were incubated for 30 min at 37 °C in 5% CO_2, after which 25 µL of lentiviral stock was added to each well (corresponding to an approximate MOI of 5). Twenty-four hours post-transduction, the medium was replaced with 500 µL fresh growth medium containing puromycin dihydrochloride (2.5 µg/mL; Gibco, A1113803). Puromycin selection was maintained until all cells in the non-transduced control wells were eliminated, with selection medium replaced daily. Following selection, puromycin was removed and surviving cells were expanded for further experiments. All lentiviral procedures were conducted under biosafety level 2 (BSL-2) conditions recommended for 3rd generation lentiviral systems.

CYP17A1, CYP19A1, CYP21A2 Enzyme Activity Assays

Enzyme activity was assessed as described previously[10–12]. HEK293T and HEK293T POR knockout cells carrying lentivirus-derived CYP17A1 or CYP21A2 or CYP19A1 were seeded in 12-well plates (0.5×10^6 cells/well) and were allowed to attach overnight under standard culture conditions.

For CYP21A2[11] and CYP17A1[10] hydroxylase activity assays, cells were incubated with 10,000 cpm [^{14}C]-progesterone and 1 µM unlabeled progesterone per well for 1 h. Radiolabeled steroids were extracted from the medium, as previously described[10], using a 1:1 mixture of ethyl acetate and isooctane (Carl ROTH) and separated by thin-layer chromatography (TLC) on silica-gel plates (Supelco®, 1009330001). TLC plates were exposed to a phosphor screen and visualized using a Typhoon™ FLA-7000 PhosphorImager. Radioactive signals were quantified with ImageQuant™ TL software (Cytiva), and enzyme activity was calculated as the percentage of product radioactivity relative to total radioactivity in the sample.

For CYP17A1 17,20-lyase and CYP19A1 aromatase activity assays, cells were incubated with 50,000 cpm radiolabeled substrate and 1 µM unlabeled steroid per well for 4 h. [^{3}H]-17α-hydroxypregnenolone was used to assess CYP17A1 17,20-lyase activity, and the formation of

^{3}H-labeled acetic acid was quantified[10]. [^{3}H]-1β-androstenedione was used as substrate for the aromatase (CYP19A1) tritiated water release assay[12].

Following incubation, culture media were collected and mixed with charcoal–dextran suspension (final compositions are 5% charcoal, 0.5% dextran, v/w/w), incubated for 5 min at room temperature, and centrifuged (5,000 × g, 5 min). The supernatant (0.5 mL) was transferred to scintillation vials containing scintillation fluid (Carl ROTH, 1P1C.1), and radioactivity was measured using a MicroBeta2 plate counter (PerkinElmer).

Western Blotting

To assess protein expression, cell pellets were incubated at 4 C° in lysis buffer (1 mM EDTA, 20 mM Tris, 150 mM NaCl, 1% (v/v) Triton X-100)[13] supplemented with protease inhibitor cocktail (Thermo Scientific, A32963) for 1 hour and centrifuged at 12,000 × g for 20 min and 4 °C[11]. Protein concentrations were determined using the Bio-Rad DC Protein Assay (5000112) according to the manufacturer's instructions. For the gel loading, equal amounts of protein (25 µg) were mixed with Laemmli buffer (50 mM Tris HCl , pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), denatured at 95 °C for 5 min, and separated by SDS–PAGE (GenScript M00659; BIO-RAD, 1658004) in Tris-MOPS-SDS running buffer (GenScript, M00138).

Proteins were transferred onto PVDF membranes (Millipore, IPFL85R) using transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). Membranes were blocked with 5% (w/v) non-fat dry milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween-20, pH 7.5) and incubated overnight at 4 °C with primary antibodies against POR (rabbit polyclonal), CYP21A2 (mouse), CYP17A1 (chicken polyclonal), or CYP19A1 (chicken polyclonal) (GenScript). After washing with TBST, membranes were incubated for 1 h at room temperature with secondary antibody (IRDye®, LI-COR Biosciences, 926-68073, 926-32218, 926-68072) diluted as 1:10,000 in 5% milk/TBST. Protein bands were detected using a Fusion FX imaging system (Vilber) at 680 nm or 780 nm, and band intensities were quantified to compare protein expression levels between wild-type and knockout cells.

Steroid Profiling

Steroid concentrations were measured using liquid chromatography–high-resolution mass spectrometry (LC–HRMS) as previously described[14].

NCI-H295R cells and POR knockout clones (T1-4 and T1-6) were seeded in 12-well plates at a density of 0.5 × 10^{6} cells per well in 1 mL growth medium. For the 96 h condition, cells were cultured for 48 h, after which 1 µM pregnenolone (Sigma-Aldrich) was added directly to the culture medium without medium replacement, and cells were incubated for an additional 48 h. For the 48 h condition, cells were seeded and cultured for 48 h, followed by replacement of the culture medium with fresh medium containing 1 µM pregnenolone, and incubated for an additional 48 h. Media samples (500 µL) were collected, and steroids were extracted, separated, and quantified according to the protocol described in reference.

Statistical analysis

Statistical analyses were performed using RStudio (RStudio, PBC). Data are presented as mean ± standard deviation (SD) unless otherwise indicated. Comparisons between two groups were performed using an unpaired two-tailed Student's t-test. For experiments involving multiple groups, statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Supplementary Figures

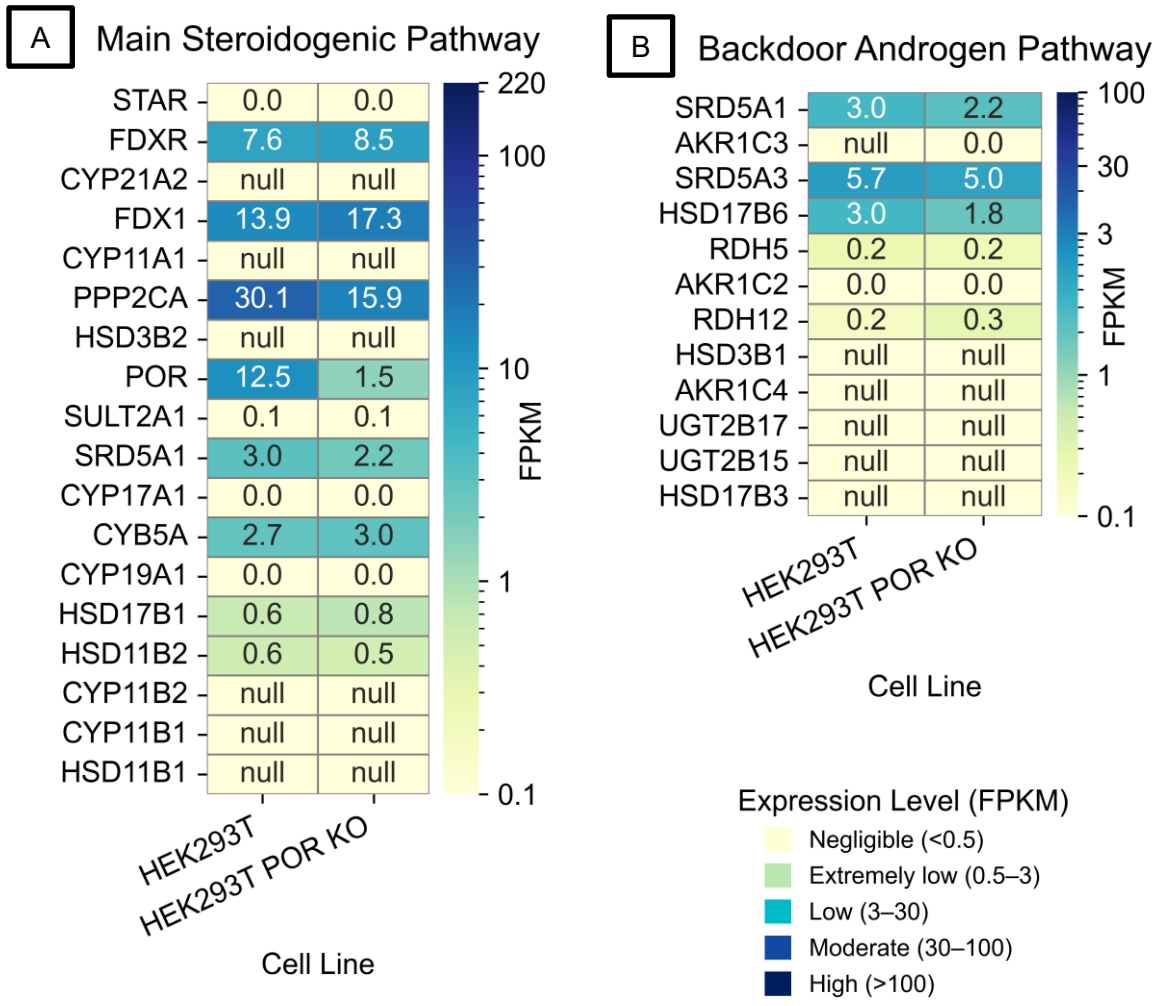


Fig S1. RNA-seq analysis of steroidogenic gene expression in HEK293T cells with POR knockout. Heatmaps display RNA-sequencing–derived gene expression levels (FPKM) of enzymes associated with the backdoor steroidogenic pathway (B) and the classical steroidogenic pathway (A) in wild-type HEK293T cells and CRISPR/Cas9-generated POR knockout (POR-KO) HEK293T cells. Absolute FPKM values are shown by color intensity and annotated numerically within each cell; Null indicates that no transcripts were detected for the corresponding gene, whereas values reported as 0.0 FPKM indicate that transcripts were detected, but their normalized expression rounds to zero at one-decimal precision. Expression levels are categorized as negligible (<0.5 FPKM), extremely low (0.5–3), low (3–30), moderate (30–100), or high (>100), as summarized in the legend.

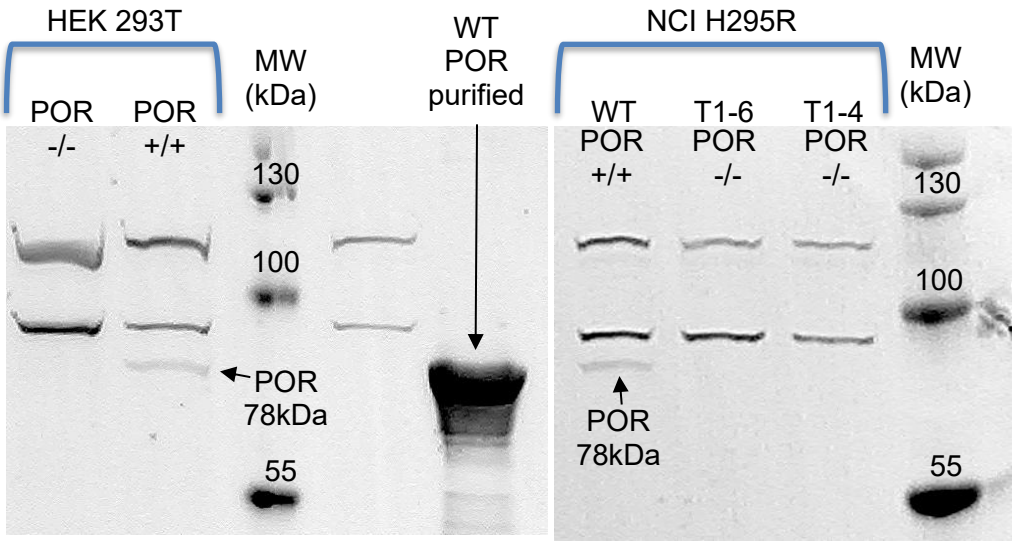


Fig S2: Western blot validation of POR expression in wild-type and POR-deficient HEK293T and H295R cells. POR protein expression was assayed by western blotting in HEK293T and NCI-H295R cell models. The cells (1 x 10^6) cells were lysed as described in Materials and Methods section and 15 ug of total protein were subjected to analysis. A band corresponding to POR protein (~78 kDa) is detected in wild-type HEK293T and H295R cells. In HEK293T POR knockout cells, this band is absent, confirming loss of POR protein expression. In NCI-H295R cells, the POR band is clearly detected in wild-type cells, whereas both independent POR knockout clones (T1-6 and T1-4) show no POR signal. Purified wild-type POR protein served as a positive control, and molecular weight markers are shown in kDa.**

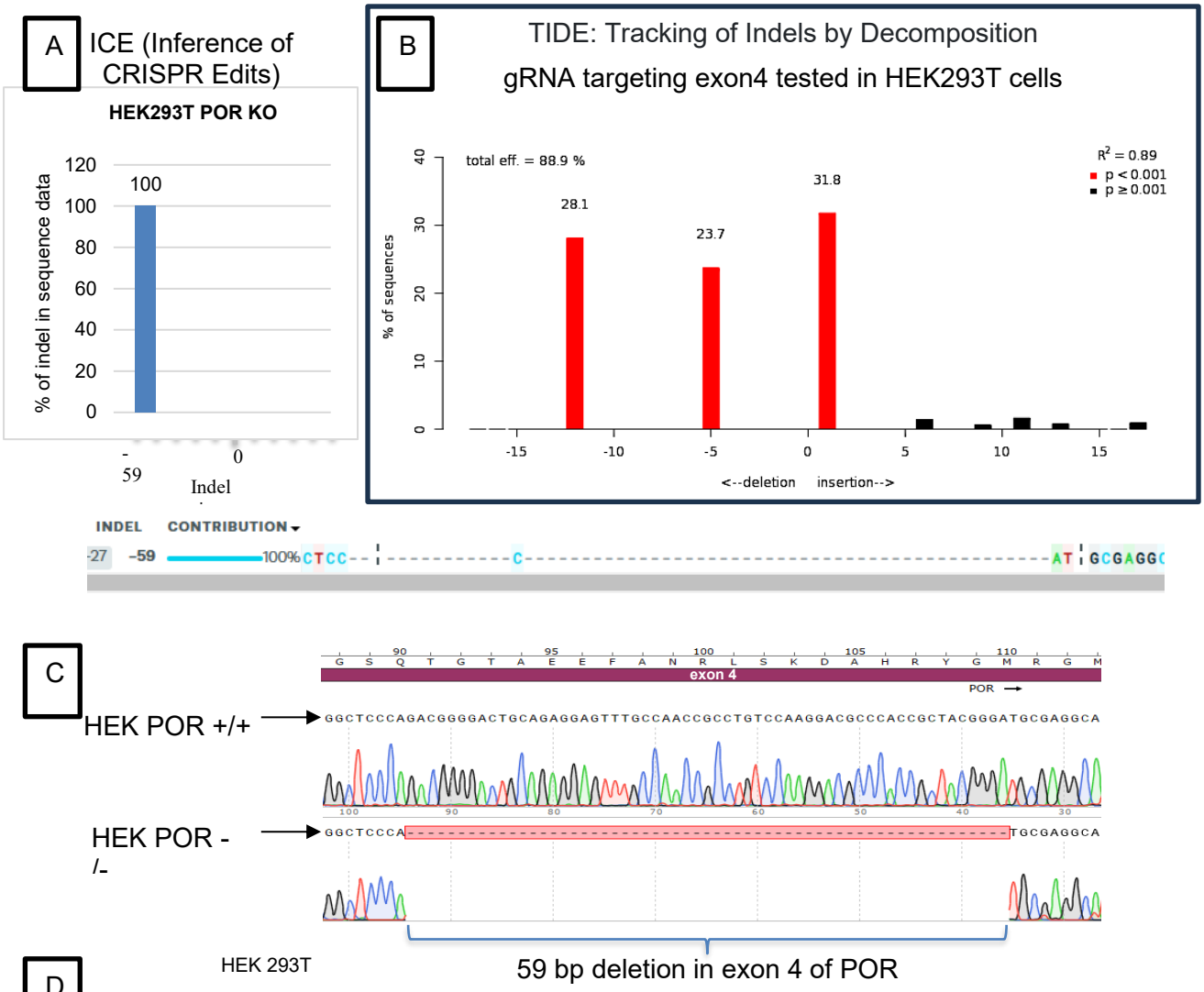


Fig S3. Validation of CRISPR/Cas9-mediated POR knockout in HEK293T cells by indel decomposition, Sanger sequencing, and PCR fragment analysis.
(A) ICE (Inference of CRISPR Edits) analysis of Sanger sequencing data from the HEK293T_POR_KO cells. ICE identified a single dominant editing outcome - a 59 bp deletion in the targeted POR site in the exon 4. The ICE decomposition assigned 100% contribution to this indel, indicating that the clone is highly homogeneous and consistent with biallelic disruption by the same deletion event.

(B) TIDE (Tracking of Indels by Decomposition) analysis was used to evaluate the overall activity of the gRNA targeting exon 4 of POR in the bulk-edited HEK293T cells before clonal isolation. TIDE estimated a total editing efficiency of 88.9% and resolved multiple indel classes generated at the CRISPR/Cas9 cut site. The most abundant events included deletions of 11 bp (28.1%) and 5 bp (23.7%), together with a 1 bp insertion (31.8%), while several lower-frequency insertion products were also detected. These results indicate highly efficient cleavage and repair by error-prone end joining at the targeted exon 4 site. (C) Representative Sanger sequencing chromatograms of wild-type and edited HEK293T cells confirmed the presence of a 59 bp deletion in exon 4 of POR in the knockout clone relative to the wild-type sequence. (D) DNA gel electrophoresis of PCR amplicons spanning the edited region further verified the deletion by showing the expected size shift from 997 bp in wild-type HEK293T cells to 938 bp in the edited clone.

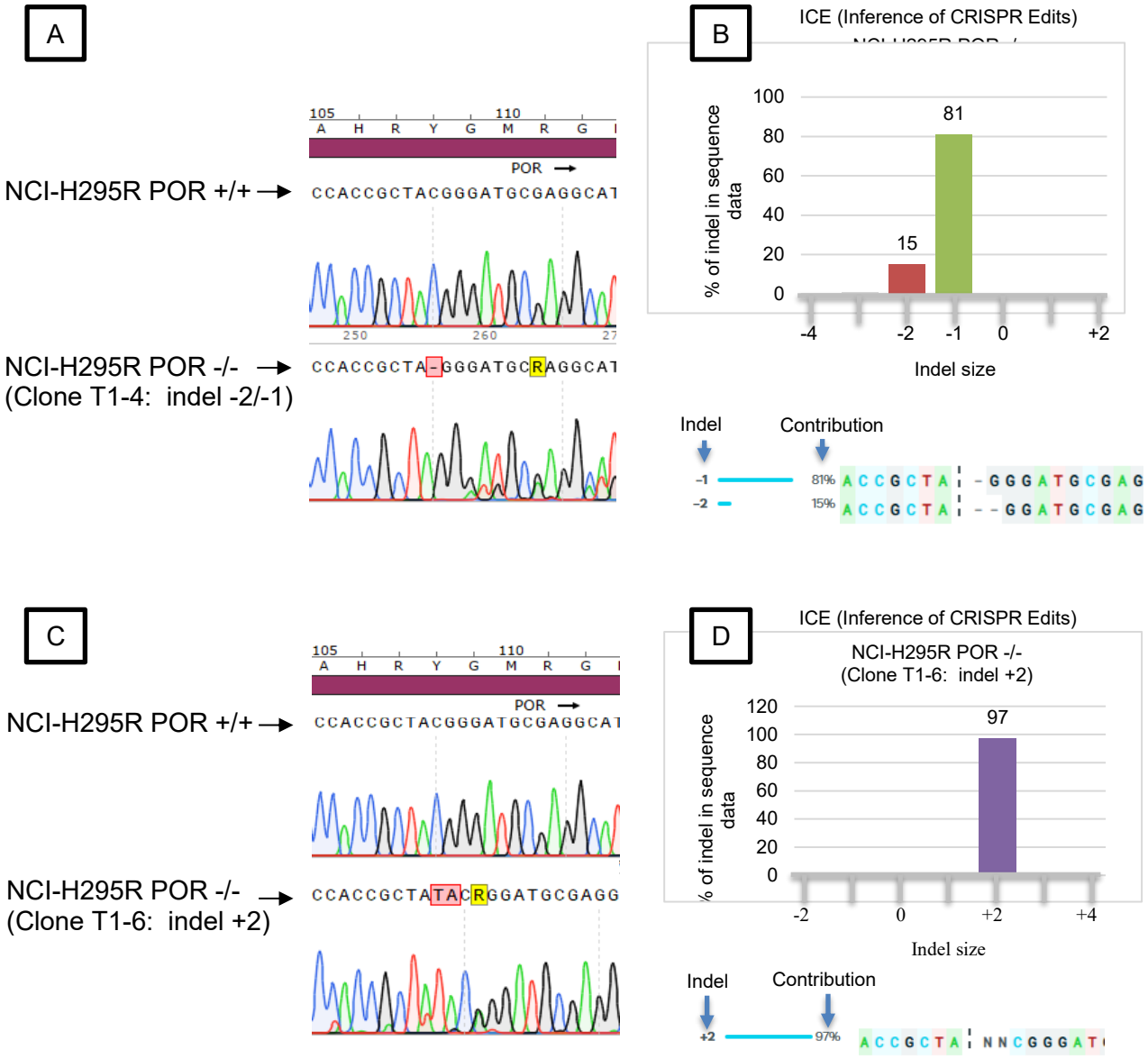
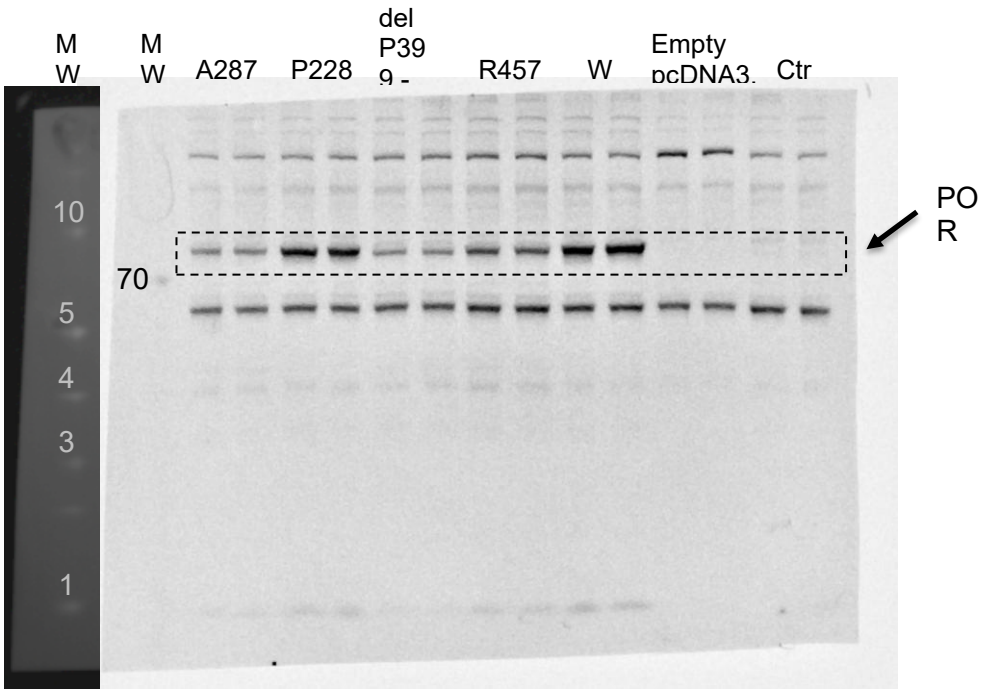


Fig S4. Molecular characterization of CRISPR/Cas9-edited POR alleles in NCI-H295R cells.
(A) Sanger sequencing chromatograms of wild-type NCI-H295R cells and the edited clone T1-4 show indels at the targeted *POR* locus. Clone T1-4 carries a mixed editing pattern consistent with compound heterozygous disruption of the two alleles.
(B) ICE (Inference of CRISPR Edits) analysis of clone T1-4 resolved two major indel classes, a -1 bp deletion and a -2 bp deletion from the predicted cut site, contributing 81% and 15% of the sequence signal, respectively, indicating a compound heterozygous genotype designated indel -2/-1.
(C) Representative Sanger sequencing chromatograms of wild-type NCI-H295R cells and the edited clone T1-6 show a distinct insertion event at the targeted *POR* locus.
(D) ICE analysis of clone T1-6 identified a predominant +2 bp (from the predicted cut site) insertion accounting for 97% of the sequence signal, consistent with a largely homogeneous edited genotype designated indel +2.

Together, the Sanger sequencing traces and ICE decomposition confirm successful CRISPR/Cas9-mediated editing of the *POR* locus in two independent NCI-H295R knockout clones, with clone T1-4 carrying compound heterozygous deletion alleles and clone T1-6 carrying a predominant 2 bp insertion.



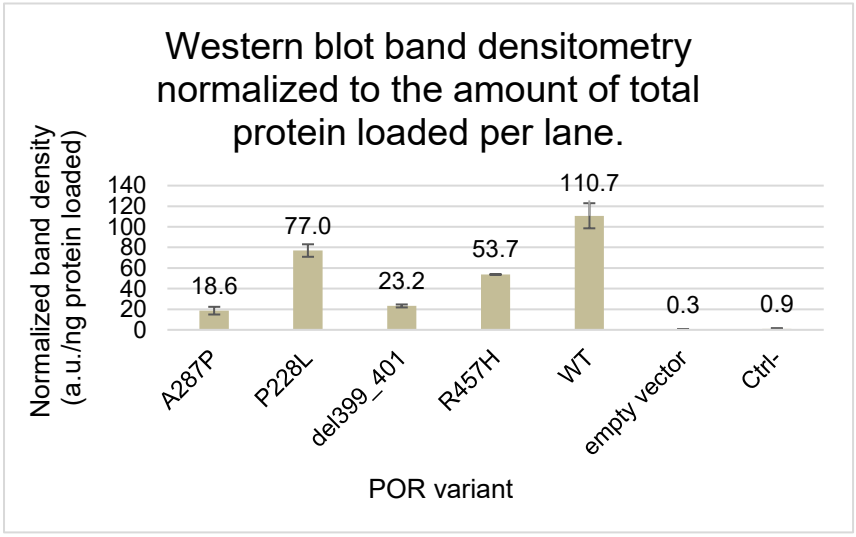


Fig S5. Expression of recombinant POR variants in POR-knockout HEK293T cells.
(A) Western blot analysis of POR-knockout HEK293T cells transiently transfected with pcDNA3.1 constructs encoding His-tagged POR variants or wild-type POR. Recombinant POR protein was detected with an anti-His antibody as a band at approximately 70 kDa (boxed region, arrow). Empty vector and non-transfected control cells showed no specific signal.
(B) Densitometric quantification of the POR bands shown in panel A. Band intensities were normalized to the total protein amount loaded per lane (25 µg) and are presented as normalized band density. Data are shown as mean ± SD.

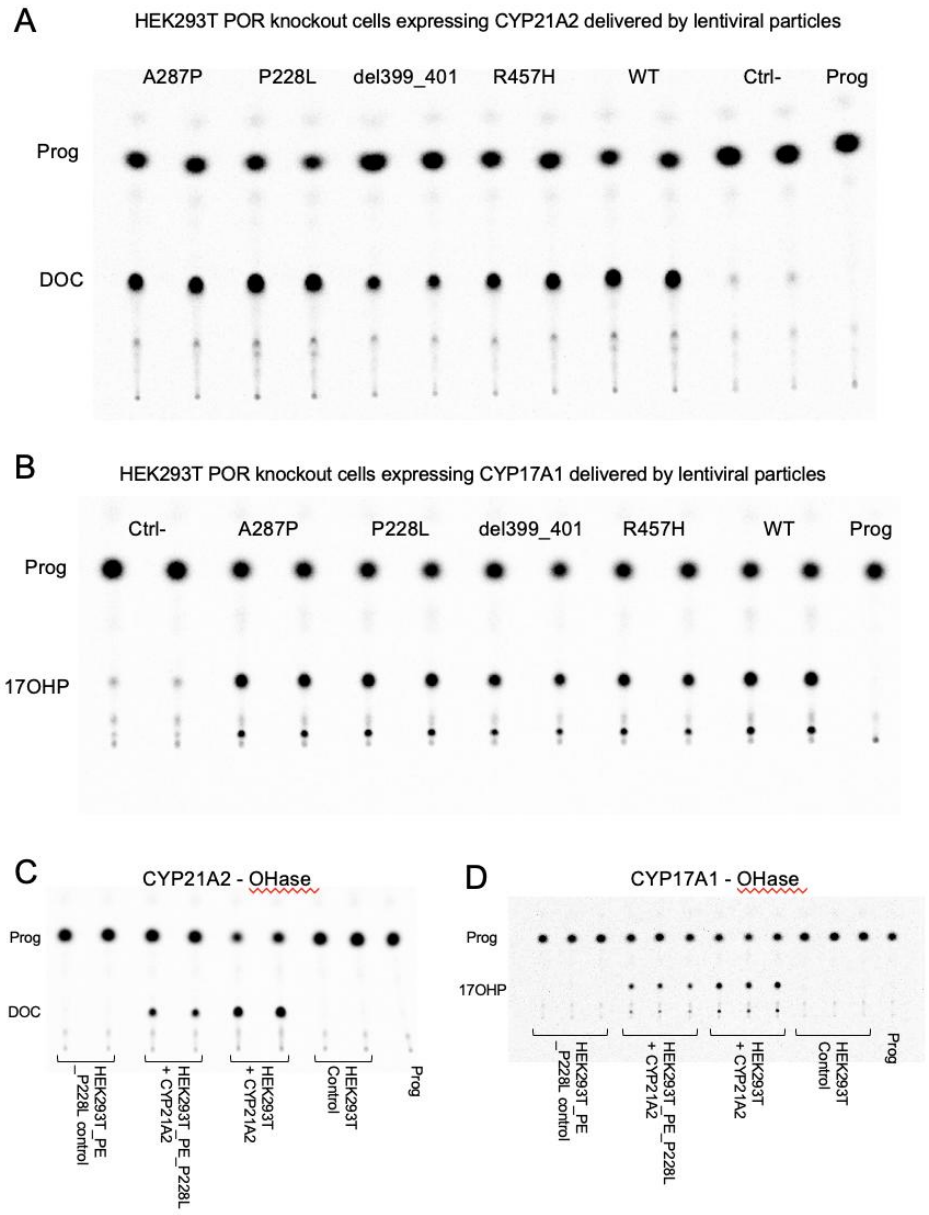


Fig S6. Functional analysis of POR variants in POR-knockout HEK293T cells co-expressing steroidogenic CYP enzymes.
Thin-layer chromatography (TLC) analysis of **(A)** CYP21A2 activity and **(B)** CYP17A1 hydroxylase activity in POR-knockout HEK293T cells transiently expressing the indicated POR variants together with lentivirally delivered CYP21A2 or CYP17A1. Progesterone (Prog) served as the substrate in both assays. Enzymatic activity was evaluated by measuring conversion of Prog to 11-deoxycorticosterone (DOC) in the CYP21A2 assay and to 17-hydroxyprogesterone (17OHP) in the CYP17A1 assay. Product formation was observed in cells expressing WT and mutant POR proteins, whereas no detectable conversion was seen in the negative control (Ctrl−). **(C)** Representative validation of CYP21A2-dependent hydroxylase activity and **(D)** CYP17A1-dependent 17α-hydroxylase activity in the P228L prime-edited HEK293T model. DOC or 17OHP productions were detected only in the presence of CYP21A2 or CYP17A1 accordingly, whereas P228L control cells and HEK293T prime-editing controls without CYPs 450 showed no detectable product formation. Prog standard was included to indicate substrate migration.

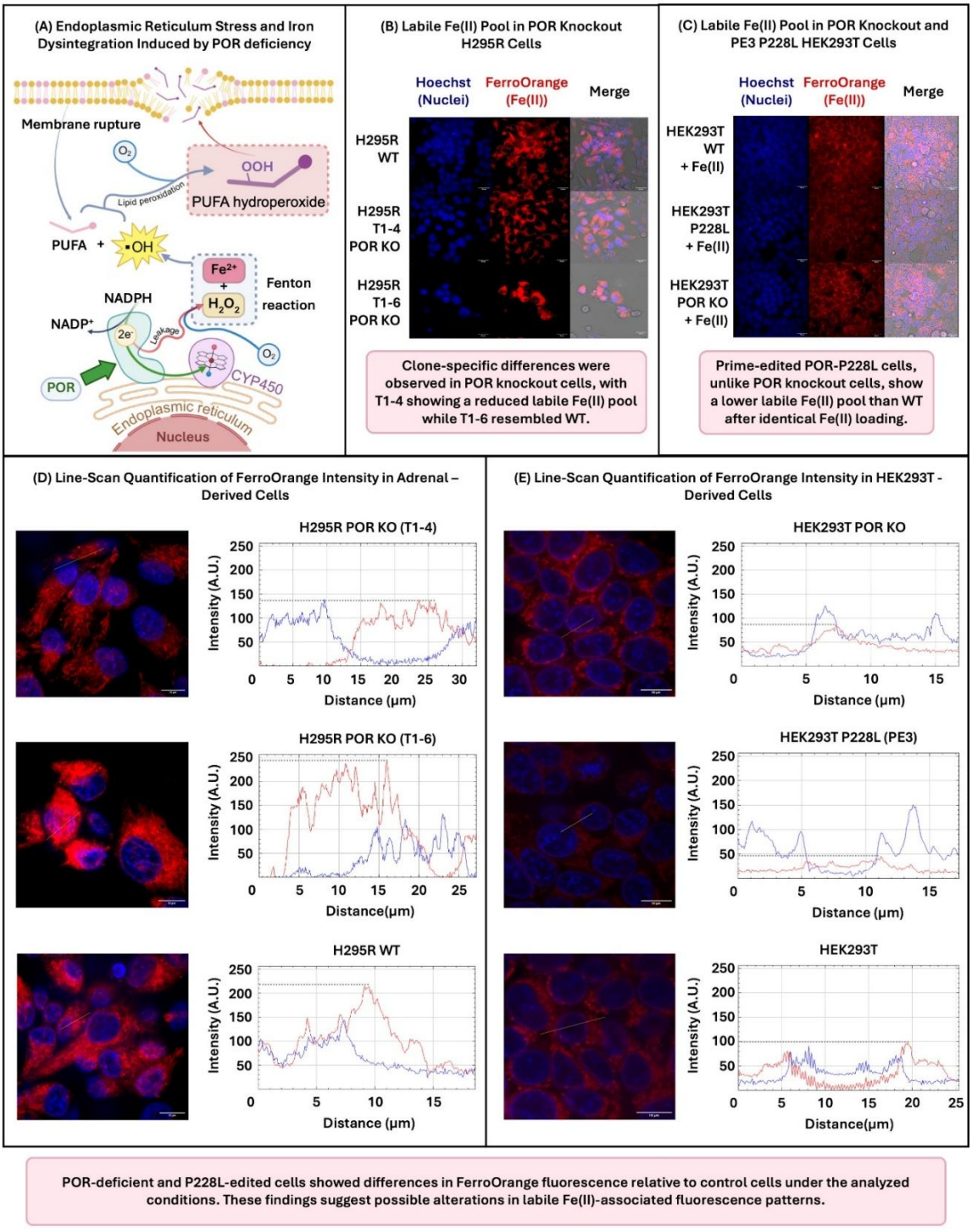


Figure S7. Altered endoplasmic reticulum redox balance and iron homeostasis. (A) Immunoblots confirming the complete eradication of POR protein in the NCI-H295R and HEK293T CRISPR/Cas9 generated knockout lines. **(B)** Schematic representation of endoplasmic reticulum stress, where uncoupled electron leakage from defective or absent POR drives reactive oxygen species (ROS) generation, Fenton chemistry, and subsequent lipid peroxidation. **(C)** Live-cell confocal microscopy utilizing FerroOrange (red) to selectively probe the labile intracellular Fe(II) pool alongside Hoechst (blue) nuclear staining in WT vs. T1-4 knockout H295R clones. **(D)**

Confocal imaging of intracellular Fe(II) pools in WT vs. Prime-Edited P228L HEK293T cells following ammonium iron(II) sulfate loading. **(E)** Specificity validation via confocal imaging following treatment with 2,2′-bipyridine (BPY), a high-affinity Fe(II) chelator, confirming the abrogation of the FerroOrange signal. **(F)** Line-scan intensity plots quantifying the fluorescence distribution, demonstrating a significant adaptive downregulation of the labile iron pool in the uncoupled mutant lines to mitigate ferroptotic susceptibility.

Dataset S1-S5 (separate file). Excel file containing, **S1** Raw RNA-seq count matrix for all samples, **S2**: Full FPKM table for NCI-H295R WT, T1-4, and T1-6.; **S3**: Full FPKM table for HEK293T WT and HEK293T POR-KO; **S4**: Differential expression table: comparison, gene ID, gene symbol, base mean/counts, log2FC, P value, adjusted P value/FDR; **S5**: LC-HRMS steroid quantification: analyte, clone, time point, replicate, concentration, protein normalization, mean, SD/SEM, P value.

SI References

1. Concordet, J.-P. & Haeussler, M. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res.* **46**, W242–W245 (2018).
2. Labun, K. *et al.* CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res.* **47**, W171–W174 (2019).
3. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
4. Menzorov, A. G., Lukyanchikova, V. A., Korablev, A. N., Serova, I. A. & Fishman, V. S. Genome editing using CRISPR/ Cas9 system: a practical guide. *Vavilov J. Genet. Breed.* **20**, 930–944 (2016).
5. Brinkman, E. K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* **42**, e168–e168 (2014).
6. Happi Mbakam, C., Rousseau, J., Tremblay, G., Yameogo, P. & Tremblay, J. P. Prime Editing Permits the Introduction of Specific Mutations in the Gene Responsible for Duchenne Muscular Dystrophy. *Int. J. Mol. Sci.* **23**, 6160 (2022).
7. Mathis, N. *et al.* Machine learning prediction of prime editing efficiency across diverse chromatin contexts. *Nat. Biotechnol.* **43**, 712–719 (2025).
8. Paquet, D. *et al.* Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature* **533**, 125–129 (2016).
9. Anzalone, A. V. *et al.* Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149–157 (2019).
10. Sharma, K. *et al.* Effect of Essential Oil Components on the Activity of Steroidogenic Cytochrome P450. *Biomolecules* **14**, 203 (2024).
11. Prado, M. J. *et al.* Characterization of Mutations Causing CYP21A2 Deficiency in Brazilian and Portuguese Populations. *Int. J. Mol. Sci.* **23**, 296 (2021).

12. Lephart, E. D. & Simpson, E. R. [45] Assay of aromatase activity. in *Methods in Enzymology* vol. 206 477–483 (Elsevier, 1991).
13. Cohen, M. *et al*. In Silico Structural and Biochemical Functional Analysis of a Novel CYP21A2 Pathogenic Variant. *Int. J. Mol. Sci.* **21**, 5857 (2020).
14. Andrieu, T., Du Toit, T., Vogt, B., Mueller, M. D. & Groessl, M. Parallel targeted and non-targeted quantitative analysis of steroids in human serum and peritoneal fluid by liquid chromatography high-resolution mass spectrometry. *Anal. Bioanal. Chem.* **414**, 7461–7472 (2022).