

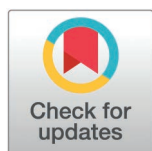
RESEARCH ARTICLE

A *VPS33B* CRISPR knockout study: In vitro evidence of an adhesion defect

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Data availability statement: The RNA-seq datasets generated and analysed during the current study are available in the Gene Expression Omnibus (GEO) repository [Link: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE298726> ; Accession number: GSE298726].

Abstract

VPS33B is a ubiquitously expressed regulator of vesicular membrane fusion and protein sorting involved in a broad range of cellular functions from organelle biogenesis to the establishment of apicobasal polarity. Loss-of-function mutations in *VPS33B* cause arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome, a rare autosomal recessive disorder with multi-organ involvement, including a characteristic proximal tubular dysfunction in the kidney. While *VPS33B* has been studied in several cell types, its role in proximal tubular epithelial cells remains poorly understood. To investigate its function, a proximal tubular cell line (RPTEC-TERT1) was CRISPR-edited to generate *VPS33B* knockout (KO) cells. These cells were characterised using bright-field imaging, immunostaining, RNA sequencing, and cell detachment assays, revealing a distinct ‘peeling’ phenotype and altered adhesion properties. Transcriptional profiling indicated changes in genes linked to cell adhesion. Together, these findings offer preliminary evidence that loss of *VPS33B* impairs cell–matrix attachment and reveal the first insights into the role of *VPS33B* within proximal tubular epithelial cells.

Introduction

Arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome is a fatal autosomal recessive syndrome caused by a germline mutation in vacuolar protein sorting-associated protein 33B (*VPS33B*) or *VPS33B* interacting protein, apical-basolateral polarity regulator, spe-39 homolog (*VIPAS39*) [1,2]. ARC syndrome is characterised by renal proximal tubular dysfunction, congenital joint contractures, and cholestasis. There are currently no curative treatments, and most patients die within the first year of life despite supportive care [3].

Approximately 75% of ARC syndrome cases are associated with mutations in *VPS33B*, and the remaining 25% with mutations in *VIPAS39* [1,4]. These pathogenic variants are distributed throughout both genes, usually resulting in loss of protein function, primarily through reduced expression and/or altered subcellular localisation of the encoded proteins [4–8].

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VPS33B is a member of the Sec1/Munc18 family of proteins and plays a key role in vesicular membrane fusion. In hepatocytes, VPS33B is essential for apical protein sorting and maintenance of polarity [9]. In hematopoietic stem cells, it regulates exosome secretion and stemness [10], while in keratinocytes and platelets it is involved in the development of specialised lysosome-related organelles (such as lamellar bodies and α -granules) [11,12]. Meanwhile in a mouse collecting cell line VPS33B has been shown to have a role in post-translational collagen modifications [13]. These findings highlight the diverse functional roles of VPS33B, which appear to be highly cell type-specific.

Despite the fact that proximal tubular dysfunction (renal Fanconi syndrome) is a cardinal feature of ARC syndrome, little is known about the role of VPS33B in renal proximal tubular epithelial cells. To date, no studies have functionally characterised the effect of VPS33B deficiency in this cell type. To address this gap, we generated and characterised a *VPS33B* knockout in the human proximal tubular epithelial cell line RPTEC-TERT1 with the aim to generate hypotheses on the role of VPS33B specifically in proximal tubular epithelial cells.

Methods

Human proximal tubular cell line (RPTEC-TERT1) culture

RPTEC-TERT1s were developed by transfecting normal human adult male primary proximal tubular cells with the human *TERT-1* gene [14]. RPTEC-TERT1 cells (ATCC, #CRL-4031™) were cultured in DMEM:F12 Medium (ATCC® 30–2006™), supplemented with hTERT RPTEC Growth Kit (ATCC® ACS-4007™) (1% Supplement A and 1.6% Supplement B), 2% FBS, and 0.1 mg/mL Geneticin™ Selective Antibiotic (G418 Sulfate) (Thermo Fisher, #10131035). Cells were cultured at 37°C with 5% CO₂. Media was changed every 2–3 days. Upon reaching 70–80% confluency RPTEC-TERT1 cells were detached using 0.05% trypsin/EDTA solution, centrifuged at 250 x g for 5 minutes and resuspended at 1:5 density.

CRISPR-Cas9D10A nickase gene editing in RPTEC-TERT1 cells

RPTEC-TERT1 cells (1.05×10^5 cells/well) were seeded into 6-well plates and transfected with a mixture of 97 μ L pre-warmed Opti-MEM, 3 μ L FuGENE® 6 Transfection Reagent, and 1 mg/well of either VPS33B Double Nickase Plasmid Set 1 (h1) (Santa Cruz, #sc-406200-NIC) or VPS33B Double Nickase Plasmid Set 2 (h2) (Santa Cruz, #sc-406200-NIC-2) [15]. Both plasmid sets contained two plasmids coding for Cas9n (D10A) double nickase, a single guide RNA (sgRNA) sequence (S1 Table), and green fluorescent protein (GFP). After 48 hours, GFP-positive, propidium iodide-negative cells were isolated by FACS to identify transfected RPTEC-TERT1 cells with a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems).

Sorted cells were collected on ice into pre-prepared 15-mL tubes containing 500 μ L of fresh medium and transferred to 24-wells, cultured, and expanded. Once the cells had been split into two 6-well plates and reached confluency, media was aspirated from one plate, each well was washed with DPBS and the plate was frozen

at -20°C for DNA extraction for the T7EI assay. The cells from the other plate were passaged into a T25 flask, cultured until 70% confluent, and then single-cell sorted by FACS into individual 96-wells. These cells were cultured for 4–6 weeks without media changes. Once the clones reached confluency, they were split into two new 96-well plates; one for DNA extraction for Sanger sequencing and one for clonal expansion.

T7 Endonuclease (T7EI) assay

To determine whether a deletion had been created in *VPS33B*, the T7EI assay was used. DNA was extracted from non-transfected and transfected cells, using the GeneJet Genomic DNA Purification kit (Thermo Fisher, #10410450). A 50- μL PCR reaction was then carried out using the human *VPS33B* primers designed to flank the sgRNA cutting sites (S2 Table). Then, a 19 μL reaction, composed of 10 μL PCR product, 2 μL 10X NEBuffer™ 2 (Biolabs, #B7002S), and 7 μL MilliQ water, was prepared. The PCR products in this solution were then melted in a thermocycler using the following conditions; 95°C for 5 minutes, ramp down to 85°C at $-2^{\circ}\text{C}/\text{second}$, ramp down to 25°C at $-0.1^{\circ}\text{C}/\text{s}$, and hold at 4°C . 1 μL of T7 Endonuclease I (Biolabs, #M0302S) was added to the reaction and incubated at 37°C for 1 hour. The T7 cleavage products were then visualised using gel electrophoresis. Successful cleavage of the *VPS33B* gene was indicated by the presence of bands at lower molecular weights than the intact PCR product.

DNA extraction and clonal genotyping

Next, DNA was extracted from the 96-well of CRISPR-transfected clones with QuickExtract™ DNA Extraction Solution (Lucigen, #QE0905T). PCR amplification of the edited region was carried out using the KAPA2G Fast Hot Start and human *VPS33B* primers (S2 Table).

Off-target PCR

To screen for off-target mutations in the transfected clones, PCRs were carried out for four top predicted off-target primer sets. These off-target sites were chosen by screening both sgRNA sequences on COSMID: CRISPR Search with Mismatches, Insertions and/or Deletions setting the maximum number of indels as 2 and allowing for 1 nucleotide insertion or deletion [16] and the WGE CRISPR Finder Tool [17]. Potential off-target sites identified by both softwares were selected. Three of these off-target regions were all found in non-coding regions of different chromosomes (Chr20:22233139–22233161, Chr13:82330988–82331010, Chr4:10167787–10167809) while the fourth (Chr1:101898463–101898485) was found to be in the olfactomedin 3 (*OLFM3*) gene. Primer sequences recommended by COSMID were used (S3 Table).

PCR clean-up & sequencing

For PCR clean up, MicroCLEAN (microzone, #2MCL-50) was used according to the manufacturer protocol. For off-target primers which generated multiple PCR products, so the QIAquick Gel Extraction Kit (QIAGEN, #28704) was used to extract the bands of interest from the gels according to the manufacturer protocol. Target genomic loci were then Sanger sequenced by Source Bioscience. Sequenced files were then analysed using the ICE Analysis tool from Synthego [18]. To understand the result of mutations found during sequencing on protein translation, VectorBuilder's DNA Translation Tool was used.

Protein extraction and Western blotting

Proteins were extracted using RIPA lysis buffer (Merck, #20–188) supplemented with cOmplete™ EDTA-free protease inhibitor (Merck, #04693132 001). Lysates and Precision Plus Protein standards (#1610377, Bio-Rad) were separated on 4–15% Mini-PROTEAN® TGX™ gels (Bio-Rad, #4561084) and transferred to 0.2 μm PVDF membranes (Bio-Rad, #1704156) using the Trans-Blot® Turbo™ system (Bio-Rad, #1704150). Membranes were blocked with 5% non-fat milk

in PBS-T (0.1% Tween-20), then incubated with primary antibodies against VPS33B (Proteintech, #12195–1-AP, 1:2,000) and GAPDH (Merck, #MAB374, 1:40,000), followed by HRP-conjugated secondary antibodies (goat anti-rabbit, #P0448, 1:1,000; rabbit anti-mouse, #PO260, 1:4,000). Detection used Pierce ECL substrate (Cytiva, #RPN2232) and imaging with a Bio-Rad ChemiDoc™ system. Membranes were stripped between probeds with Re-Blot Plus Strong Solution (Millipore, #2504). Densitometry was performed on ChemiDoc™ tiff files using FIJI with background subtraction.

RNA-seq of RPTEC-TERT1 gene edited cells

RPTEC-TERT1 Control, KO1, and KO2 clones were cultured to 70–80% confluency before RNA extraction with the RNeasy Plus Mini kit (QIAGEN, #74136). Four biological replicates were processed for sequencing for each clone. RNA integrity was assessed (RIN > 7.0) using the Qubit RNA BR assay alongside Agilent's 4200 TapeStation. Library preparation was performed with the Watchmaker RNA Library Prep Kit with Polaris™ Depletion (product number BK0002–096). Sequencing was performed on the NextSeq 2000 platform from Illumina. Sequencing was conducted on the NextSeq 2000 instrument at a concentration of 800 pM, utilizing a 57-bps-end run with corresponding 8-bps unique dual sample indexes and unique molecular indexes (UMIs).

Initial bioinformatic analysis and quality control of FASTQ files was carried out using the nf-core/rnaseq pipeline v3.12.0 [19]. Downstream analysis was carried out on R version 4.3.1. DESeq2 v1.42.1 was used to normalise read counts and carry out the differential gene expression analysis. For further quality control analyses, principal component analyses (PCA) and heatmaps were plotted and generated with pheatmap v1.0.12, DESeq2 [20]. The default Wald test was used to compare the Control and Knockout samples. Approximate posterior estimation for generalised linear model (GLM) coefficients was used to shrink log-fold changes between samples. For gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, genes with an adjusted p-value of <0.05 and $|\log_2 \text{fold change}| > 0.26$ were analysed using clusterProfiler v4.10.1 [21] and gprofiler2 v0.2.3 [22], respectively. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) [23] was used to investigate protein-protein interactions (PPIs) between adhesion-associated differentially expressed genes (DEGs) and to generate graphs of enriched Reactome pathways [24] and WikiPathways [25]. PPI modules were generated with Molecular Complex Detection (MCODE) [26] with default settings (degree cut-off = 2, node score cut-off = 0.2, and K-core = 2). Graphs and plots were generated with enrichplot v1.22.0, ggplot2 v3.5.1, and Cytoscape v3.10.4 [27].

RPTEC-TERT1 monolayer survival assay

A monolayer survival assay was conducted by seeding RPTEC-TERT1 cells in 96-well wells at 5×10^4 cells/well in RPTEC-TERT1 media. Every day for 11 days of culture, the wells were imaged using an EVOS M5000 microscope at x4 and x10 magnification and monolayer 'survival' (intact monolayer) or 'failure' (peeling) was recorded.

Immunofluorescence staining for adhesion complexes (Phospho-paxillin) in RPTEC cells

RPTEC-TERT1 cells (5×10^4 /well) were seeded in 96-well plates and cultured for 6 days. Cells were fixed with 4% PFA, blocked (10% FBS, 1% BSA, 0.1% Tween-20 in PBS), and permeabilized with 0.1% Triton-X in PBS. Cells were stained with anti-phospho-paxillin antibody (Abcam, #ab4832, 1:100), Alexa Fluor™ Plus 647 anti-rabbit secondary antibody (Thermo Fisher, #A32795, 1:200), TRITC-phalloidin (Merck, #90228, 1:1000), and DAPI (Merck, #90229). Imaging was performed on an Axio Observer 7 microscope (Zeiss, #491917-0001-000) and analysed with CellProfiler v4.2.6. Phospho-paxillin-positive punctae were enhanced and isolated using EnhanceorSuppressFeatures and IdentifyPrimaryObjects functions. To investigate phospho-paxillin-F-actin association, phalloidin staining was diluted using the DilateImage function with a disk-shaped structuring element of size 12 and co-localisation of phospho-paxillin-punctae was quantified per image.

Cell detachment assay

A cell detachment assay was adapted from a previously published protocol [28]. RPTEC-TERT1 cells were seeded at 1×10^5 cells/well in a 96-well plate, with 11 wells seeded for each timepoint (0–10 minutes). After overnight culture, the wells were washed with DPBS and placed in an oven heated to 37°C to maintain a stable temperature. 100 μ L of 0.05% trypsin-EDTA pre-warmed to 37°C was added to each well, except time-point 0, where DPBS was added. At 1-minute intervals, trypsin was removed and media added to stop trypsinization. Wells were then washed, fixed with 4% PFA for 10 minutes, and stained with 2.3% crystal violet for 15 minutes.

The plate was then washed under running water and left to dry on paper towels overnight. Then, 50 μ L/well of 10% acetic acid was added to dissolve the crystal violet followed by 250 μ L of distilled water. Absorbance was read at 570 nm on a plate reader (BioTek, Synergy HT). Cell adhesion was calculated as a percentage relative to the 0-minute timepoint, after subtracting background control absorbance (cell-free well treated with crystal violet).

Cell adhesion assay

RPTEC-TERT1 cells were trypsinised and seeded at 1×10^5 cells/well in a 96-well plate. These cells were allowed to attach overnight. Then, the cells were washed with DPBS three times before being fixed with 50 μ L/well of 4% PFA at room temperature for 10 minutes. Then, crystal violet staining was carried out as described above.

Statistical analysis

All statistical analyses were carried out on GraphPad Prism Software v10.3.1. The normal distribution of the data was confirmed with a Shapiro-Wilk test. For normally distributed data, two-tailed t-tests or one-way ANOVAs with Tukey's multiple comparisons test were used. For survival analyses a log-rank test was used. Quantitative data, displayed as bar and line charts, are expressed as means $\pm$ standard error of the mean (SEM; error bars). Data are reported to 2 significant figures.

Results

Generation of a human renal proximal tubule VPS33B-KO cell line

CRISPR-Cas9n double nickase technology was used to target mutations to the first exon of the *VPS33B* gene in RPTEC-TERT1 cells. Two *VPS33B* double nickase plasmid sets (h1 and h2), targeting an overlapping region at the start of exon one, were compared for their cutting efficiency using the T7EI assay. The h2 plasmids had a higher cutting efficiency than h1 as evidenced by the brighter 350 bp band and minimal wild-type *VPS33B* 650 bp band generated (Fig 1A; S1 Fig). Thus, the following experiments used h2-transfected cells.

Following CRISPR transfection, cells were fluorescence-activated cell sorted into 96-well plates to initiate clonal cell cultures. Clones were then expanded and genotyped. As ARC syndrome is an autosomal recessive disorder, a homozygous *VPS33B*-KO clone was desired. Sanger sequencing revealed that 2/8 selected clones were heterozygous, with insertions of either 3 bp or 14 bp in one allele. 3/8 clones were compound heterozygotes with the same 18 bps and 33 bps deletions (Fig 1C – blue). A further 2/8 clones were homozygous with 56 bp deletions in both alleles (Fig 1C – red), while a final clone contained no edits in *VPS33B* and is referred to from here on as the Control clone.

Vector Builder's DNA translation tool predicted that the heterozygous 18 and 33 bps deletions would result in 6- or 11-amino acid deletions, respectively. Meanwhile, the 56-bps homozygous deletion was predicted to cause a frameshift resulting in a nonsense mutation and premature stop codon at amino acid 30 of the *VPS33B* sequence (Fig 1D). As the 56-bps homozygous deletion was predicted to essentially prevent protein translation, the two clones carrying this mutation were selected as the KO clones (KO clone 1 and KO clone 2).

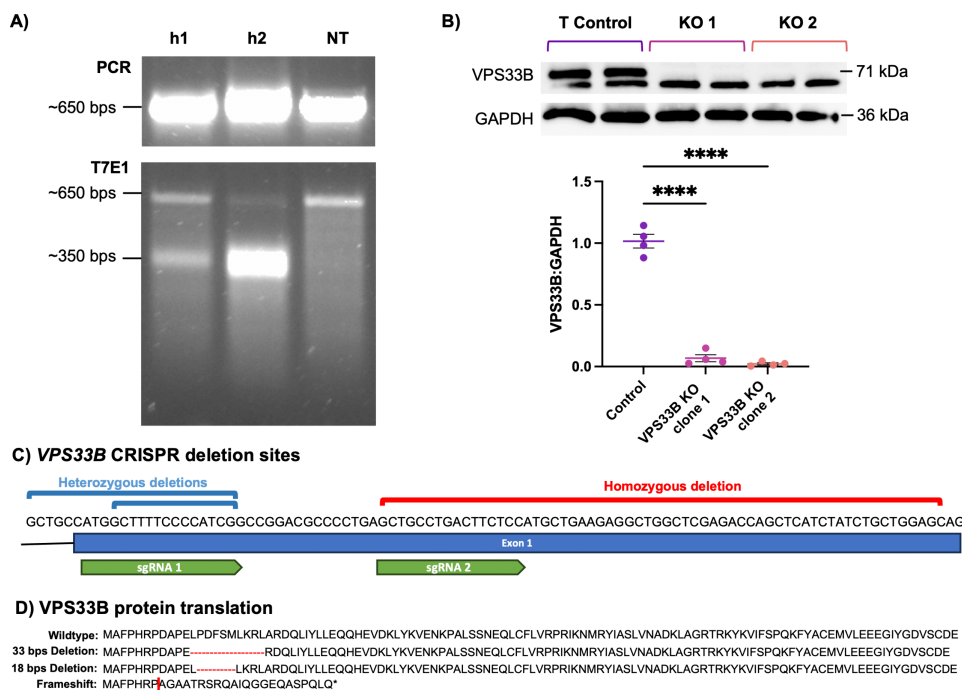


Fig 1. Generation and validation of VPS33B-knockout in a human renal proximal tubule epithelial cell (RPTEC) line. A) Initial PCR reaction and subsequent T7E1 assay carried out on gDNA collected from plasmid h1 transfected (left), plasmid h2 (middle), or untransfected (NT; right) RPTEC-TERT1 cells. B) Western blot (n=4) analysis of VPS33B expression in clones of a human proximal tubule cell line (RPTEC-TERT1). Data represents mean $\pm$ SEM. **** $P < 0.0001$. ANOVA. Tukey's multiple comparisons test. C) Sequence of 5' of the first exon of the VPS33B gene and the deletions (compound heterozygotes – blue, homozygotes – red) identified in CRISPR-treated RPTEC-TERT1 cells. Exon 1 and the CRISPR single-guide RNAs (sgRNAs) were depicted as a blue rectangle and green labels, respectively. D) Predicted changes to the amino acid sequence of VPS33B resulting from the mutations in B. Red dashes represent amino acid deletions and red line represents frameshift site.

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Densitometry showed that VPS33B was significantly reduced in KO clone 1 (93% reduction, Dunnett's test, $p < 0.0001$) and KO clone 2 (98% reduction, Dunnett's test, $p < 0.0001$) compared to the Control (Fig 1B; S1 Fig). Thus, the Western blotting demonstrated that the premature stop codon in the KO clone prevents VPS33B protein translation.

Finally, to explore the extent of off-target gene editing, likely off-target sites were predicted using the COSMID: CRISPR Search with Mismatches, Insertions and/or Deletions tool [16] and the WGE CRISPR Finder Tool. The top four predicted regions were Sanger sequenced, but no deletions or insertions were detected at these sites in either the Control line or the KO clones.

Transcriptomic analysis of a VPS33B-KO RPTEC-TERT1 cell line

VPS33B is involved in a range of processes which differ from cell type to cell type and no specific information is available on its role in the proximal tubule. Thus, transcriptomic analysis of the RPTEC-TERT1 clones was used to identify potential mechanisms which may underlie the proximal tubular dysfunction seen in ARC syndrome.

Principal component analysis (PCA) demonstrated clear clustering of the knockout clones along PC1, accounting for 65% of the variability in the data (Fig 2A). Hierarchical clustering was carried out to generate pairwise comparisons of the expression profiles of the samples. This demonstrated that the KO clones clustered together, separately from the Control (Fig 2B). Furthermore, correlation coefficients observed in this hierarchical clustering analysis showed high correlation (>0.998) in all pairwise comparisons, suggesting no outlying samples (Fig 2B). To reduce noise and increase the power

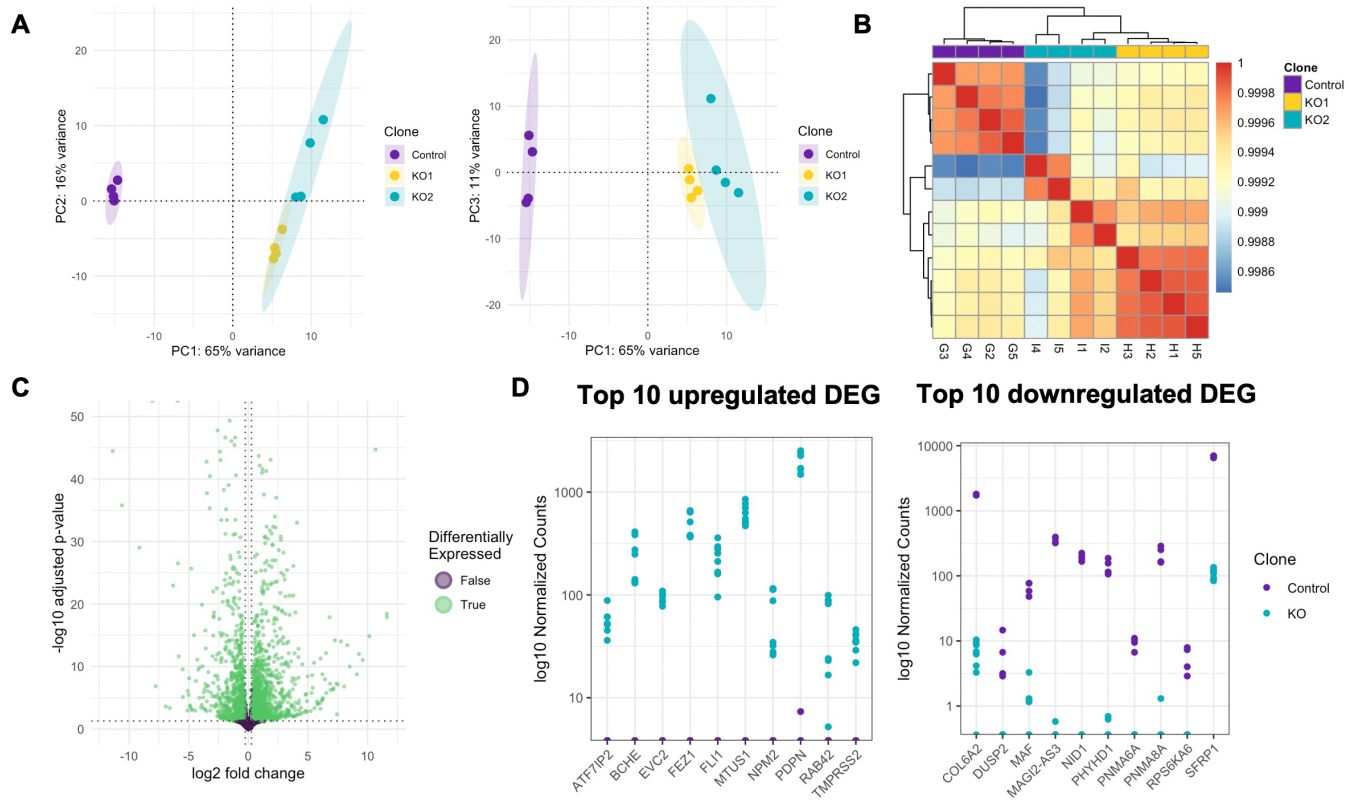


Fig 2. Exploratory transcriptomic analyses of Control and VPS33B-knockout RPTEC-TERT1 cells. A) PCA of Control and knockout transcriptomes. B) A heatmap comparing the similarity of the c=Control samples (G2-5) to the two knockout clones (KO1: H1-5 and KO2: I1-5). C) A volcano plot of differentially expressed genes between Control and knockout clones ($P < 0.05$, $|\log_2 \text{fold change}| > 0.26$). False: purple, True: green. D) Plot of log₁₀ normalised counts of top 10 up- and down-regulated (by fold-change) DEGs. Abbreviations: DEG: Differentially expressed genes, PCA: Principal component analysis.

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of the differential gene expression analysis, subsequent analyses were carried out comparing the Control line against the two *VPS33B*-KO clones pooled. Analysis of differential gene expression showed a high number of differentially expressed genes (DEGs) with relatively low fold-changes. Thresholds of adjusted $p < 0.05$ and $|\log_2 \text{fold change}| > 0.26$ were selected. With this fold-change threshold, a total of 3,362 genes were found to be differentially expressed with 1,790 being upregulated and 1,572 being downregulated in *VPS33B*-KO samples compared to the Control samples (Fig 2C).

The top 10 upregulated DEGs, ranked by largest fold change were: *ATF7IP2* ($\log_2 \text{FC} = 9.27$), *BCHE* ($\log_2 \text{FC} = 11.6$), *EVC2* ($\log_2 \text{FC} = 10.1$), *FEZ1* ($\log_2 \text{FC} = 12.8$), *FLI1* ($\log_2 \text{FC} = 11.6$), *MTUS1* ($\log_2 \text{FC} = 13.2$), *NPM2* ($\log_2 \text{FC} = 9.59$), *RAB42* ($\log_2 \text{FC} = 9.11$), *PDPN* ($\log_2 \text{FC} = 10.7$), and *TMPRSS2* ($\log_2 \text{FC} = 8.50$) (Fig 2D). The top 10 downregulated DEGs were: *COL6A2* ($\log_2 \text{FC} = -8.06$), *DUSP2* ($\log_2 \text{FC} = -6.93$), *MAF* ($\log_2 \text{FC} = -6.32$), *MAGI2-AS3* ($\log_2 \text{FC} = -11.4$), *NID1* ($\log_2 \text{FC} = -13.0$), *PHYHD1* ($\log_2 \text{FC} = -9.16$), *PNMA6A* ($\log_2 \text{FC} = 7.78$), *PNMA8A* ($\log_2 \text{FC} = -10.6$), *RPS6KA6* ($\log_2 \text{FC} = -6.60$), and *SFRP1* ($\log_2 \text{FC} = -5.94$) (Fig 2D).

To understand the functional roles of the DEGs in the *VPS33B*-KO cells, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were carried out to investigate the DEGs' association to biological processes and identify enriched pathways, respectively. GO and KEGG analyses each retrieved 503 and 273 significantly enriched terms (adjusted p -value < 0.05). To investigate the most significantly enriched terms, the top 20 (by adjusted p -value) GO and KEGG terms were investigated.

Both the top 20 KEGG and top 20 GO terms showed enrichment of cell adhesion-associated pathways (GO: “positive regulation of cell adhesion”; KEGG: “Focal adhesion” and “ECM-receptor interaction”). The top 20 GO terms also included 11 terms associated with kidney development or development in general (“kidney development”, “renal system development”, “renal tubule development”, “nephron development”, “renal epithelium development”, “nephron tubule development”, “embryonic organ development”, “renal tubule morphogenesis”, “nephron epithelium development”, “kidney morphogenesis”, and “epithelial tube morphogenesis”) and two terms associated with ossification (“ossification” and “osteoblast differentiation”) (Fig 3A, 3B). Meanwhile, in addition to the cell adhesion-associated terms, the KEGG analysis showed enrichment of cancer associated pathways (“Pathways in cancer”, “MAPK signaling pathway”, “PI3K-Akt signaling pathway”, “Human papillomavirus infection”, “Proteoglycans in cancer”, “Rap1 signaling pathway”, “Hippo signaling pathway”, “Transcriptional misregulation in cancer”, “Hepatocellular carcinoma”, “Ras signaling pathway”, “Gastric cancer”), and infection associated pathways (“Efferocytosis”, “Salmonella infection”) in the top 20 KEGG terms (Fig 3C).

As both the top 20 KEGG and GO terms mentioned cell adhesion, adhesion-associated DEGs were further explored. Among the GO terms enriched in our analysis, eight were associated with cell-substrate adhesion (“positive regulation of cell adhesion”, “cell-substrate adhesion”, “regulation of cell-substrate adhesion”, “substrate adhesion-dependent cell spreading”, “cell adhesion mediated by integrin”, “positive regulation of cell-substrate adhesion”, “regulation of cell adhesion mediated by integrin”, and “negative regulation of cell adhesion”). The DEGs associated with these terms were isolated, yielding 209 adhesion-associated DEGs (S4 Table). Exploration of the top 30 adhesion-associated DEG showed a downregulation of *NID1*, *SFRP1*, *TNFRSF14*, *NPNT*, *EGR3*, *ECM2*, *ABI3 BP*, *SERPINE2*, *ITGA2B*, *EBI3* and an upregulation in *PDPN*, *IL1B*, *PIK3 CG*, *LCK*, *SELP*, *PREX1*, *IL1A*, *DTX1*, *LIMS2*, *CAMSAP3*, *RASGRP1*, *GREM1*, *ANGPT1*, *GLI2*, *ERBB3*, *CHRD*, *MDK*, *SAA1*, *SPOCK2*, and *CD1D* in VPS33B-KO cells compared to Controls (Fig 3D). Further Reactome Pathways and WikiPathways analyses of the 209 adhesion-associated DEGs highlighted enrichment of terms associated with extracellular matrix organisation, integrin cell surface interactions, and focal adhesions (S2A, S2B Fig). A PPI analysis yielded 7 modules of protein interactions associated with extracellular matrix organisation, development, and Semaphorin-Plexin and Ephrin-Eph signalling (S2C Fig; S5 Table).

General phenotypic characterisation of VPS33B-KO RPTEC-TERT1 cell line in extended culture

Next, for an initial phenotypic characterisation of VPS33B-KO RPTEC-TERT1 clones, RPTEC-TERT1 cells were cultured in a 96-well plate for 11 days and observed daily with brightfield microscopy. At 1 day following seeding, all clones appeared elongated (blue arrows in Fig 4A–4C). By day 3 of culture, cells reached confluency and by day 5, they reached the typical epithelial cobblestone shape expected of RPTEC-TERT1 cells [29]. No obvious differences in cell behaviour were noted between Control and KO clones until day 6 of culture, when VPS33B-KO but not Control cells were seen to progressively begin to ‘peel’ as a monolayer from the tissue culture plastic (black arrows, Fig 4B, 4C).

To quantify this peeling phenotype, RPTEC-TERT1 cells were cultured for up to 11 days and the number of days taken for each clone to peel from the tissue culture plastic was counted for 7 independent replicates. This was used to calculate the probability of monolayer maintenance over time with a peeling event constituting monolayer “failure”. While the Control line did not peel during the 11-day period studied, both of the VPS33B-KO lines peeled completely within 11 days (Log-rank test for trend, $p < 0.0001$; Fig 4D). Most VPS33B-KO cells peeled at 7 days post seeding while some wells remained unpeeled for up to 10 days. Brightfield imaging showed that RPTEC-TERT1 cells appeared elongated initially upon seeding and became smaller and more regular after reaching confluency around day 3 of culture. Both VPS33B-KO clones began peeling around day 7 after which elongated cells invaded the space left behind by the peeled monolayer (blue arrows, Fig 4B, 4C).

Cell attachment assays

To investigate the mechanobiological mechanism underlying the monolayer peeling observed in the KO clones, cell attachment and cell detachment assays were carried out. To investigate whether the VPS33B KO reduced the ability

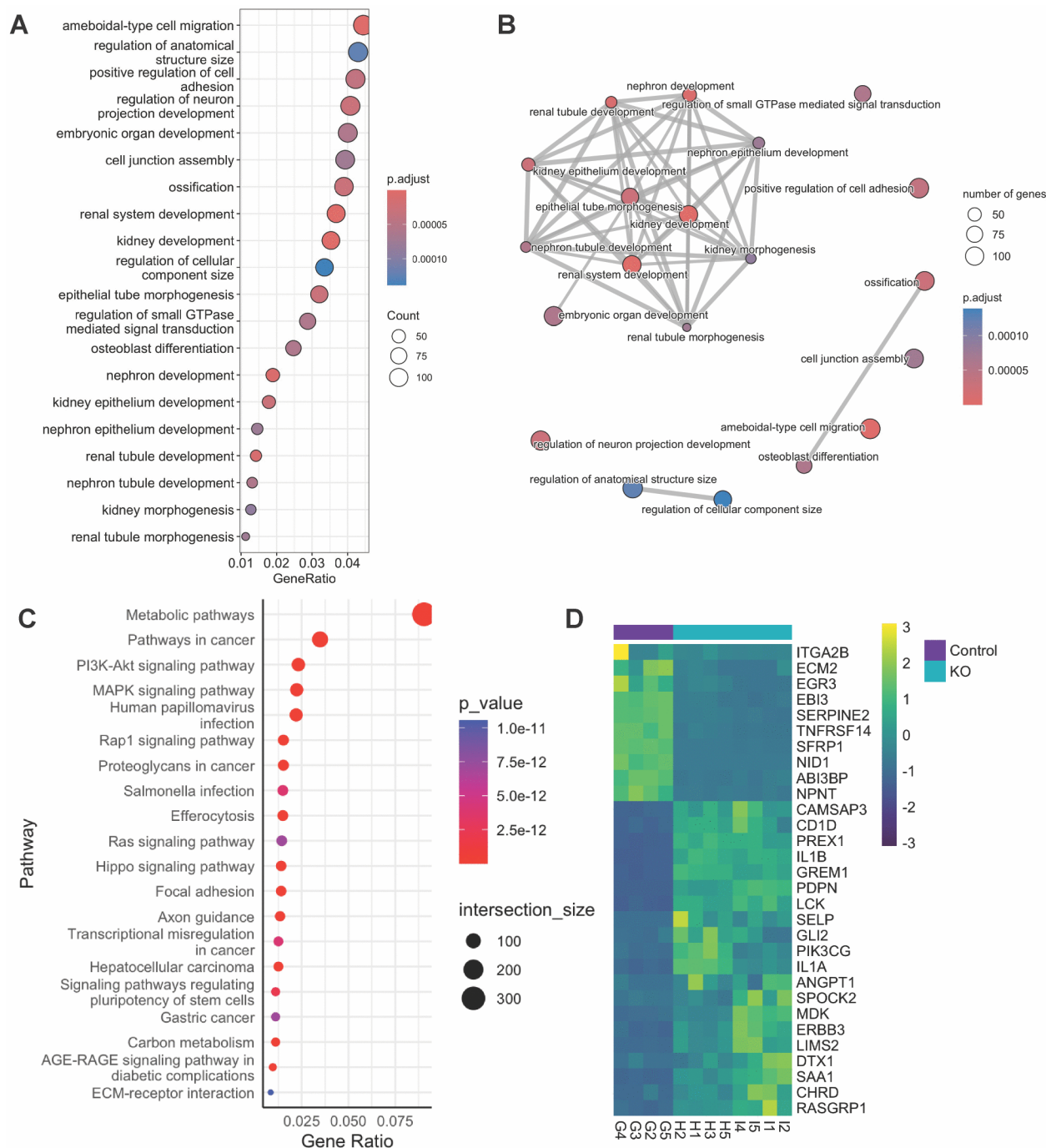


Fig 3. Pathway analysis of VPS33B-knockout RPTEC-TERT1 cells. A) Dot plot and B) enrichment map of the top 20 enriched GO terms (by adjusted p-value) comparing Control to knockout clones. C) Top 20 enriched KEGG terms (by adjusted p-value). D) Heat map of top 30 adhesion-associated DEGs by log2FC. Abbreviations: DEG: Differentially expressed genes, GO: gene ontology.

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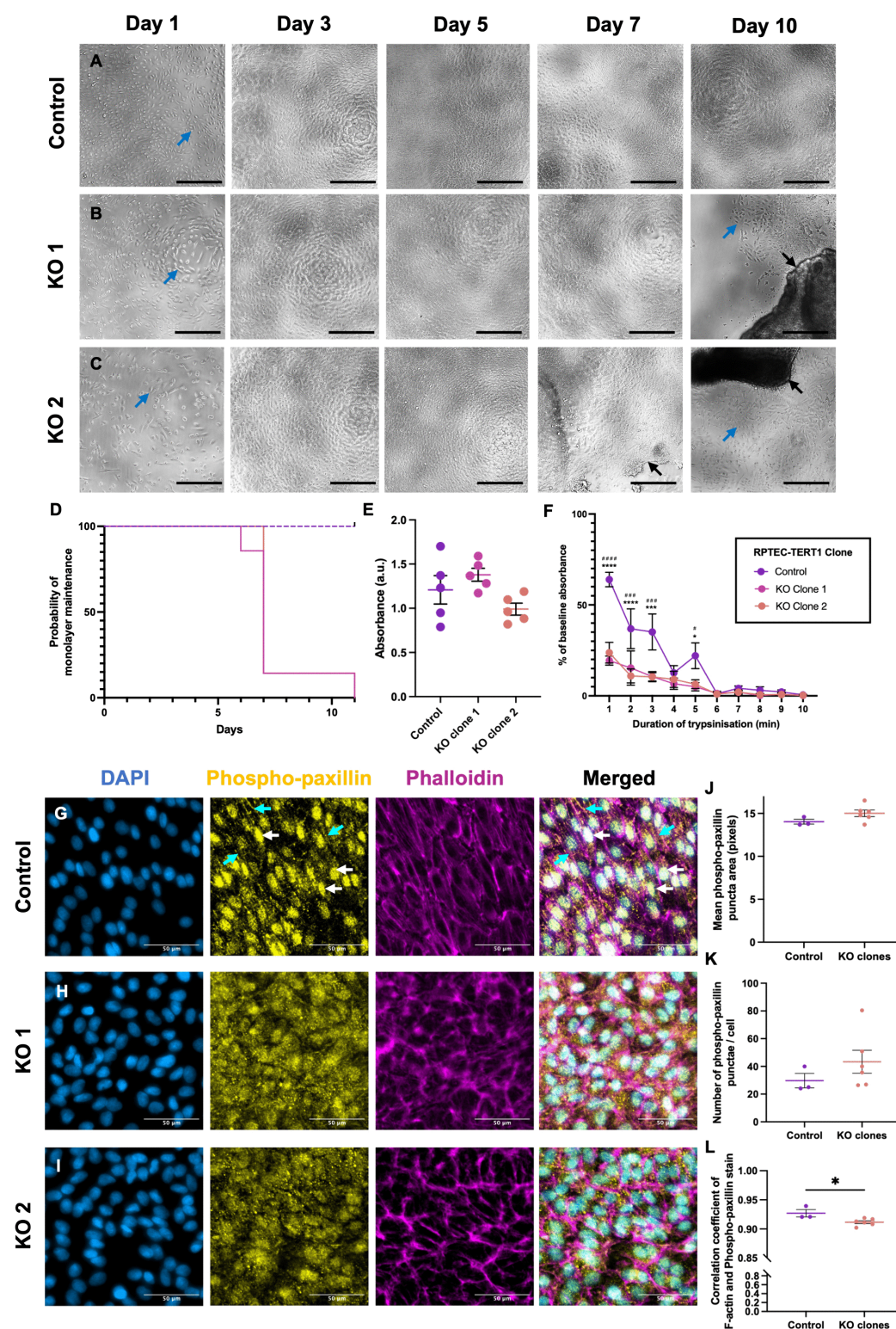


Fig 4. VPS33B-KO RPTEC-TERT1 clones demonstrate weaker attachment compared to controls. x10 brightfield images of A) Control, B) VPS33B-KO clone 1, and C) VPS33B-KO clone 2 RPTEC-TERT1 cells in 96-well plates after 1, 3, 5, 7, and 10 days of culture. Blue arrows: elongated RPTEC-TERT1 cells. Black arrow: Monolayer peeling. Scale bar: 100 μ m. D) Survival analysis for time until monolayer peeling (n = 7). Log-rank test for

trend. $p < 0.0001$. E) Analysis of absorbance of crystal violet one day after seeding. ANOVA. Tukey's multiple comparisons test ($n = 5$). F) Results of a cell detachment assay carried out on Control and VPS33B-KO RPTEC-TERT1 cells. Two-way ANOVA. Tukey's multiple comparisons test. Control vs KO clone 1: $\#p < 0.05$; $###p < 0.001$, $####p < 0.0001$. Control vs KO clone 2: $*p < 0.05$; $***p < 0.001$, $****p < 0.0001$ ($n = 5$). Representative x40 images of G) Control: CRISPR-transfected control, H) KO1: VPS33B-KO RPTEC-TERT1 Clone 1, I) KO2: VPS33B-KO RPTEC-TERT1 Clone 2 stained with DAPI (blue), anti-phospho-paxillin antibody (yellow), and phalloidin-stained F-actin (magenta). Cyan arrows indicate linear junctional staining of phospho-paxillin. White arrows indicate nuclear phospho-paxillin staining. $n = 3$. Scale bar = 50 μm . Analysis of J) mean size of phospho-paxillin-positive puncta area, K) number of punctae per cell, and L) association of phospho-paxillin-positive punctae with the actin cytoskeleton comparing Control cells to a pooled sample of VPS33B-KO clones. $n = 3$ per genotype. T-test. Data represents mean $\pm$ SEM. $*p < 0.05$. Abbreviations: a.u.: arbitrary units.

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of cells to initially adhere to their substrate upon seeding, cells were seeded into 96-well plates. The following day, they were washed with DPBS to remove any cells which had not attached and stained with crystal violet (Fig 4E). No significant differences in cells' ability to attach to the tissue culture surface were observed between the Control and KO clones (ANOVA. $p > 0.05$). To investigate this further, analysis of the strength of cell adhesion was carried out, using a cell detachment assay. Cells were seeded into a 96-well plate and allowed to attach overnight. These cells were then trypsinised for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 minutes and a crystal violet stain was used to visualise the cell density remaining (Fig 4F).

Results showed that both VPS33B-KO clones took significantly less time (both reaching $<20\%$ attachment in 1 minute) to detach compared to the transfected control (reaching $<20\%$ attachment after 6 minutes; (Fig 4F). There was no significant difference in the rate of cell detachment between the two VPS33B-KO clones at any time point, with KO clone 1 and KO clone 2 being 81% and 76% detached within the first minute of trypsinisation compared to 36% in the Control line. At minute 1 of trypsinisation a Tukey's multiple comparisons test showed significant differences in cell detachment between Control and KO clone 1 ($p < 0.0001$), and Control and KO clone 2 ($p < 0.0001$). This significant difference in the rate of trypsinisation could be observed until minute 5 of trypsinisation. Unlike the cell attachment assay, this demonstrated a clear demarcation between both Control and KO clones, suggesting that VPS33B KO results in the strength of cell-substrate adhesion.

Adhesion complex distribution is disrupted in VPS33B-KO RPTEC-TERT1 cells

To better understand the source of this attachment defect, we investigated focal adhesions in VPS33B-KO RPTEC-TERT1 cells. Knockdown of focal adhesion components, such as integrin $\beta 3$ in Chinese Hamster Ovary cells, can reduce the ability of cells to attach to substrates [30]. Thus, it was hypothesized that the adhesion defect would be associated with a change in focal adhesion component distribution.

Immunofluorescent staining with anti-phospho-paxillin antibodies and phalloidin visualised focal adhesions and actin, respectively. Control cells showed nuclear (white arrows) and cortical (cyan arrows) localisation of phospho-paxillin with punctate staining associating with the actin cytoskeleton (Fig 4G). Meanwhile, both KO lines showed more diffuse, cytoplasmic, punctate staining of phospho-paxillin with no obvious cortical localisation (Fig 4H, 4I).

To investigate whether an automated image analysis software could differentiate between Control and KO clones, CellProfiler software was used to analyse the images and the results of the Control and pooled KO clones were compared. Analysis found no significant differences in size (Fig 4J) or number (Fig 4K) of phospho-paxillin punctae between Control and KO clones. To quantify the association of the focal adhesion with the actin cytoskeleton, the co-localisation of the phospho-paxillin and phalloidin stain were quantified. This analysis demonstrated that VPS33B KO significantly reduced phospho-paxillin proximity to the actin cytoskeleton (Fig 4L; $p = 0.027$) without reducing the number or size of the phospho-paxillin punctae.

Discussion

This study generated VPS33B-KO RPTEC-TERT1 cells which displayed a phenotype consistent with impaired cell adhesion. RNA sequencing followed by KEGG and GO term enrichment analysis revealed significant dysregulation of

adhesion-associated pathways, and functional assays (spontaneous monolayer peeling and cell detachment tests) supported a cell-substrate adhesion defect.

The transcriptomic changes observed in VPS33B-deficient cells broadly mirrored patterns seen in previous VPS33B depletion models. GO analysis of differentially expressed genes uncovered changes associated with renal and epithelial development/tube formation, regulation of cell and anatomical structure size, ameboidal-type cell migration, ossification, neuronal development and cell adhesion. Meanwhile, KEGG analysis showed enrichment in ECM-receptor interactions and focal adhesions, infection-associated (HIV and HPV infections) and cancer-associated pathways. Similar pathways were found to be dysregulated in Chai et al. who examined *Vps33b^{fl/fl}; Alfp-Cre (hepatocyte-KO)* mouse livers [9,31], with their top 5 GO terms being “Cell adhesion”, “Biological adhesion”, “Defence response”, “Response to wounding”, and “Immune response”. Their top 5 enriched pathways were “ABC transporters”, “Cell adhesion”, “ECM receptor interaction”, “Toll-like receptor signalling pathway”, and “Focal adhesion” [31]. Despite the differences in species and organ, there is commonality in the enriched adhesion and inflammatory/immune GO and KEGG terms seen in Fig 3. Proteomic comparison of *Vps33b*-KO and *Vps33b*-overexpressing immortalised mouse tendon fibroblasts also showed enrichment of adhesion-related GO terms [32]. Thus, the similarity of transcriptional signatures across species and cell types strengthens the conclusion that VPS33B is involved in regulating adhesion-related pathways.

Interestingly, our GO analysis also identified enrichment of renal development-associated terms, which may reflect the known developmental renal dysfunction seen in ARC syndrome. This finding aligns with reports that some patients present with proximal tubular symptoms shortly after birth, suggesting disruption of developmental processes [33]. Although global *Vps33b*-KO mice are embryonically lethal before metanephric kidney development (E9.5) [34,35], future studies involving conditional, nephron-specific knockouts could explore the potential roles of VPS33B in developmental pathways specific to the kidney.

There is little previous literature on the role of VPS33B in cell-substrate adhesion. However, it is known that VPS33B can bind to integrins $\beta 1$ and $\beta 3$, which as part of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, are found in focal adhesions [36,37]. It has also been shown in murine cells that *Vps33b* is involved in integrin $\alpha 11\beta 3$ -mediated endocytosis and integrin outside-in signalling, which affects cell spreading [36]. It is not entirely clear what the role of VPS33B in integrin outside-in signalling is. It is unlikely to be as simple as trafficking the integrins, as similar levels of activated integrin $\alpha 11\beta 3$ were found on and *Vps33b*-KO mouse platelets in response to thrombin stimulation, meaning cells did not have problems trafficking this integrin to their cell surfaces [36]. However, our paper provides the first evidence of a direct role for VPS33B in cell adhesion.

Phospho-paxillin staining showed changes in response to *VPS33B* KO. Phospho-paxillin staining was focal adhesion-like (punctate), associating with the actin cytoskeleton in the Control clones. However, the pattern was consistently disrupted in *VPS33B*-KO RPTEC-TERT1 cells. This suggests that focal adhesion protein distribution is disrupted by *VPS33B* KO. Additionally, the lack of a significant difference in the number of phospho-paxillin-positive punctae between *VPS33B*-KO and Control cells suggests that *VPS33B* KO does not affect focal adhesion number, only their distribution to points of adhesion.

This cell-adhesion defect provides a potential mechanistic link to renal pathology in ARC syndrome. The proximal tubule receives the largest volume of fluid of all the nephron components, being the first section of the nephron after the Bowman’s capsule [38]. Therefore, it is likely to experience the highest shear stress in the nephron. We propose a model in which VPS33B loss weakens cell–matrix adhesion, compromising the ability of proximal tubule cells to resist mechanical stress *in vivo*. However, further studies using patient-derived cells or tissue samples, or *in vivo* models, are needed to confirm this hypothesis. Future studies may investigate the impact of shear stress on the *VPS33B*-KO cells generated in this study by culturing them in a perfused microfluidic system where shear stress could be specifically controlled.

Given the rarity of ARC syndrome and challenges associated with sampling renal tissue (i.e., haemorrhages following biopsies), future exploration may rely on autopsy samples or patient-derived urine epithelial cells [39]. Such studies could examine focal adhesion organization, integrin function, and mechanical stress responses to expand upon the findings presented here.

Conclusion

This study presents preliminary evidence that VPS33B contributes to cell–matrix adhesion in human renal proximal tubular epithelial cells. Characterisation of VPS33B-KO RPTEC-TERT1 cells revealed cell adhesion defects, supported by RNA-seq analysis, detachment assays, and disrupted phospho-paxillin staining patterns. These findings suggest one possible cellular mechanism by which VPS33B deficiency could impact proximal tubular function in ARC syndrome. However, given the limitations of *in vitro* modelling and the need for independent validation, further studies, particularly in primary human tissue and *in vivo* systems, will be needed to determine the relevance of these observations to ARC syndrome pathophysiology.

Supporting information

S1 Table. Plasmids used for CRISPR. 20-nucleotide sequences of target-specific sgRNA (single guide RNA) portion of plasmids used for CRISPR knockout of VPS33B (counted from the beginning of the NCBI sequence). (DOCX)

S2 Table. VPS33B primers used for generation of human RPTEC-TERT1 VPS33B-KO CRISPR clones. (DOCX)

S3 Table. Primers used off-target sequencing of human RPTEC-TERT1 VPS33B-KO CRISPR clones. (DOCX)

S4 Table. List of 209 adhesion-associated differentially expressed genes between Control and VPS33B-KO CRISPR clones. (CSV)

S5 Table. MCODE cluster analysis results. (CSV)

S1 Fig. Uncropped western blots and gels. A) VPS33B Western blot displayed in [Fig 1B](#). B) Following VPS33B staining, the membrane was washed and reblotted for GAPDH, also displayed in Figure B. GAPDH intensity was very high, preventing visualisation of the ladder and prior VPS33B stain without severe overexposure. Images were captured with Bio-Rad ChemiDoc™ system. X denotes channels not included in final blot. C) Electrophoresis gel image displayed in [Fig 1A](#) acquired using a Gel Doc EZ System. (TIFF)

S2 Fig. Pathway analysis and protein. Top 10 A) Reactome Pathway and B) WikiPathways analysis of 209 adhesion-associated DEGs. C) Protein-protein interactions between adhesion-associated DEGs displayed using Cytoscape. PPI modules (highlighted as squares and numbered) were generated with MCODE. Abbreviations: DEG: differentially expressed gene. (TIFF)

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Writing – review & editing: Maria Caluianu, Kimberley A. Owen.

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