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1 mCCD_{cl1} cells show Plasticity Consistent with the Ability to Transition between Principal and
2 Intercalated Cells

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8 **Running title:**

9 Plasticity of mCCD_{cl1} cells

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33 **Abstract**

34 The cortical collecting duct of the mammalian kidney plays a critical role in the regulation of body
35 volume, sodium pH and osmolarity and is composed of two distinct cells types, principal cells and
36 intercalated cells. Each cell type is detectable in the kidney by the localization of specific transport
37 proteins such as Aqp2 and ENaC in principal cells and V-ATPase B1 and Cx30 in intercalated cells.
38 mCCD_{c11} cells have been widely used as a mouse principal cell line on the basis of their physiological
39 characteristics. In this study, the mCCD_{c11} parental cell line and three sub-lines cloned from isolated
40 single cells (Ed1, Ed2, and Ed3) were grown on filters to assess their transepithelial resistance,
41 transepithelial voltage, equivalent short circuit current and expression of the cell-specific markers Aqp2,
42 ENaC, V-ATPaseB1 and Cx30. The parental mCCD_{c11} cell line presented amiloride-sensitive electrogenic
43 sodium transport indicative of principal cell function, however immunocytochemistry and RT-PCR
44 showed that some cells expressed the intercalated cell-specific markers V-ATPase B1 and Cx30,
45 including a subset of cells also positive for Aqp2 and ENaC. The three subclonal lines contained cells that
46 were positive for both intercalated and principal cell-specific markers. The vertical transmission of both
47 principal and intercalated cell characteristics via single cell cloning, reveals the plasticity of mCCD_{c11}
48 cells, and a direct lineage relationship between these two physiologically important cell types, and is
49 consistent with mCCD_{c11} cells being precursor cells.

50 **Introduction**

51 The collecting duct of the mammalian kidney is responsible for 4-5% of total sodium reabsorption and
52 approximately 10% of total water reabsorption from the ultrafiltrate. It plays a critical role in the
53 regulation of urine volume, pH and osmolarity, with two thirds of the hypo-osmotic fluid entering the
54 collecting duct being reabsorbed in the CCD (cortical collecting duct) (10).

55 The CCD is composed of two distinct cells types, principal cells (PCs) and intercalated cells (ICs), the
56 latter being sub-divided into α and β subtypes. Principal and intercalated cells can be distinguished by
57 morphological and immunocytochemical criteria and are functionally specialized. Principal cells reabsorb
58 water and sodium through aquaporin2 (Aqp2) and the epithelial sodium channel (ENaC) respectively, are
59 responsible for K^+ excretion and express several physiologically important genes including *Hsd11b2*. In
60 contrast, intercalated cells regulate urinary pH through V-ATPase, reabsorb K^+ through H^+/K^+ ATPase and
61 produce ATP via the Connexin 30 (Cx30) apical membrane hemichannels (33). The different cell types
62 are detectable in the kidney by immunostaining. Typically, PCs show apical membrane staining for Aqp2
63 or ENaC channels. V-ATPase localises to the apical membrane of α -ICs, and the Cl^-/HCO_3^- exchanger

64 AE1 localises to the basolateral membrane. Conversely V-ATPase localises to the basolateral membrane
65 in β -ICs.. Morphological differences include the presence of a primary central cilium on PCs while the
66 apical membrane of ICs are covered with a dense layer of microvilli (27).

67 In the mouse CCD, the ratio between PCs and ICs is approximately 70:30 (14), but the lineage
68 relationship between these cells is unclear. Although PCs and ICs appear distinct, they exhibit a degree of
69 functional overlaps and inter-regulation. For example, sodium reabsorption can occur through thiazide-
70 sensitive transport in β -IC (20) in addition to reabsorption through ENaC in PCs. Furthermore, ATP
71 released from Cx30 in ICs is an inhibitory regulator of sodium and water reabsorption via calcium
72 signaling, resulting in sodium regulation in PCs (22). Sodium, water, and potassium transport in PCs are
73 also regulated through the paracrine ATP/prostaglandin E2 (ATP/PGE2) signaling cascade involving ICs
74 (13). ICs also appear to have plasticity between α - and β -ICs under acidotic conditions or the deletion of
75 the extracellular matrix protein DMBT1 (1, 12), as well as the existence of a third IC type in the
76 collecting duct characterized by the presence of apical V-ATPase but no bicarbonate exchanger AE1 on
77 the basolateral membrane (17).

78 The ratio of principal cells to intercalated cells is influenced by multiple factors including the
79 transcription factor Adam10 and the E3 ubiquitin ligase Mib1, both of which are required for Notch
80 signaling, and the histone H3 K79 methyltransferase Dot11. The deletion of floxed alleles of *Adam 10*,
81 *Mib1* and *Dot11* via genetic crosses with *Aqp2-cre* (for Adam10^{fl/fl}) or *Hoxb7-cre* (for Dot11^{fl/fl} and Mib1^{fl/fl})
82 mice, results in a reduced number of principal cells (14, 15, 38). Whilst it is evident that activation of the
83 Notch pathway is important in determining CCD identity, the underlying mechanisms remain unclear.
84 Studies of the *Dot11*^{-/-} mice also showed that ICs lacked di-methyl K79 suggesting that the cells had
85 previously expressed *Aqp2-cre* and therefore presumed to be *Dot11*^{-/-} and originate from PC cells (37).
86 Earlier studies on primary mouse β -ICs showed they can give rise to both α -ICs and PCs, however
87 cultures of primary PCs did not appear to show the same capacity for interconversion (8). Related studies
88 on immortalized M1 cells showed a proportion of cells expressing a dual phenotype suggesting a degree
89 of cell plasticity and *in vivo* evidence for bi-potential comes from studies of *Foxi1*^{-/-} mice in which the
90 collecting ducts comprised of a single cell type that was positive for both principal and intercalated cell
91 markers (37). Finally, it has recently been reported that a subset of ureteric bud tip cells (UBTCs)
92 expressing p63 act as progenitors for cortical intercalated cells and that cell determination, at least for this
93 population of IC cells, may already be specified at this early stage of development (7). The inter-
94 relationship between PC and IC cells, both during development and in the adult, is complex and is yet to
95 be fully elucidated.

96
97 mCCD_{cl1} cells have been described as a spontaneously transformed cell line derived from a single clone,

98 which was obtained by microdissecting the cortical collecting duct of a wild type mouse (11). mCCD_{cl1}
99 cells express ENaC as well as the necessary cellular machinery, including 11 beta-hydroxysteroid
100 dehydrogenase type 2 (HSD11b2), mineralocorticoid and glucocorticoid receptors (MR and GR), to
101 enable their stimulation by physiological concentrations of aldosterone and have therefore been used as a
102 model for studying PC physiology. The mCCD_{cl1} cells have proved to be a useful tool for studying the
103 regulation of principal cell ion transporters such as ENaC (4, 30) or ROMK channels (9). In these studies,
104 mCCD_{cl1} cells have been shown to possess the functions of *in vivo* CCDs, and are therefore considered to
105 be a “highly differentiated murine principal cell line” (21). However, here we show that mCCD_{cl1} is a
106 heterogenous cell population expressing PC and IC markers Precursor cells expressing both PC and IC
107 markers are reminiscent of dual-staining transition cells, recently observed *in vivo* in the collecting duct
108 (36). Moreover, we used clonal cell sublines to show that this heterogeneity could be transmitted through
109 a single cell and that mCCD_{cl1} cells exhibit a renewable bi-potential phenotypic characteristic of precursor
110 cells.

111

112

113 **Methods**

114 *Cell culture*

115 The mCCD_{cl1} cell line was previously established and kindly provided by Bernard Rossier (University of
116 Lausanne, Lausanne, Switzerland). The cells were cultured at 37°C and 5% CO₂ in Phenol red free
117 DMEM/F12 media (Invitrogen, Life Technologies), with the following supplements: insulin (5µg/ml),
118 triiodothyronine (1nmol/l), sodium selenite (60nmol/l), dexamethasone (50nmol/l), apotransferrin
119 (5µg/ml), EGF (10ng/ml), FBS (2%), and penicillin-streptomycin (Pen-Strep, 100U/ml-100µg/ml) for
120 optimal culture conditions as previously described (11).

121 *Cloning*

122 Clonal cell lines derived from mCCD_{cl1} cells were established using the dilution method as previously
123 described (28). Briefly, confluent mCCD_{cl1} cells in growth medium were trypsinized, suspended in
124 medium, and serially diluted in a 96-well plate. Following appropriate dilution, the presence of single
125 cells was independently verified and confirmed by observing the growth of the resulting single colonies in
126 the wells over 3 days of culture. The colonies were then trypsinized and transferred to a T75 flask for
127 culture. The cloning process produced 8 clonal sub-lines, among which 3 lines (named Ed1, Ed2 and Ed3)
128 were selected for further studies based on morphological differences.

129 *Polarization and TEER Measurements*

130 Parental mCCD_{cl1} cells were used between passages 26 and 30. Parental cells and clonal sub-lines were
131 polarized by growing cells on Corning Costar™ Snapwell™ Permeable Supports inserts (12mm, 0.4 μm
132 pore size). Cells were seeded at a 1:1 split ratio and grown for 10 days. On day 8, the cells were fed with
133 basal medium containing charcoal-stripped FBS and Pen-Strep supplements only and on day 9 with basal
134 media containing Pen-Strep only. Measurements for transepithelial voltage (V_{te}) and transepithelial
135 resistance (R_{te}) were made with a transepithelial volt-ohm-meter and a set of chopstick “STX” electrodes
136 (EVOM², World Precision Instruments) and the equivalent short circuit current (I_{sc}) calculated using
137 Ohm’s law. By convention, a negative I_{sc} reflects either electrogenic secretion of cations, electrogenic
138 absorption of anions, or a combination of both. Aldosterone and amiloride (Sigma Life Science, UK)
139 were used at 3nM and 10μM respectively.

140 *Immunocytochemistry*

141 Immunocytochemistry was performed on cells cultured on the Snapwell™ permeable membranes as
142 described previously. Cells were fixed using 4% PFA for 20min. Double immunostaining of Aqp2 and
143 Cx30 was made using polyclonal goat anti-mouse Aqp2 (Abcam ab105171; 1:200) and rabbit anti-mouse
144 Cx30 antibody (Invitrogen 712200; 1:100). The fluorescent secondary antibodies were Alexa Fluor 568-
145 and 488- donkey anti-rabbit and anti-goat respectively, at a dilution of 1:500. Immunostaining for ENaC
146 and V-ATPase was conducted using a rabbit anti-mouse α-ENaC antibody (1:1000; kindly provided by
147 Prof. J. Loffing, University of Zurich, Switzerland) and a goat anti-mouse V-ATPase A1 antibody (Santa
148 Cruz Biotechnology, sc-28801; 1:50), with the fluorescent secondary antibodies described above.
149 Immunostaining for V-ATPase B1 was performed with a rabbit anti-mouse antibody (Life Technologies,
150 PA535052, 1:50), conjugated to Alexa Fluor 568. Triple immunocytochemistry was performed on the
151 parental mCCD_{cl1} cell line with the same primary antibodies for V-ATPase and α-ENaC, and the addition
152 of an anti-acetylated α-tubulin conjugated antibody at a dilution of 1:50 (Santa-Cruz Technologies, sc-

153 23950 with AF488, green secondary). Alexa Fluor 647 (far red) was used as the fluorescent secondary
154 antibodies for V-ATPase in this experiment. Immunocytochemistry for p63 and Δ Np63 was performed
155 using a rabbit anti-mouse Δ Np3 antibody and a goat anti-mouse p63 antibody (Biolegend 619001, 1:500,
156 and abcam ab114059, 1:200, respectively).

157 The permeable membranes were cut from their support using a scalpel blade, and mounted on microscope
158 slides with DAPI mounting medium (Prolong Gold antifade reagent with DAPI, Life Technologies).
159 Images were taken with a Q-Imaging camera (Canada) on a Nikon Eclipse Ti fluorescent microscope, with
160 DAPI, FITC, TRITC, and CY5 filters applied, for DAPI, Alexa fluor 568, 488, and 647 respectively.
161 Both 60X 1.4 NA Plan Apo and 40X 1.3 NA Plan Flur oil objectives were used. To visualize cell
162 polarization, cells were imaged using an Andor Revolution spinning disc microscope (Oxford
163 Instruments), with the iQ3 imaging software and the iXon EMCCD camera. The 40x 1.3NA (oil
164 immersion) objective was used. Excitation was performed at 569nm, for detection of Alexa Fluor 594
165 fluorophore (conjugated to the α -ENaC antibody). Cell autofluorescence was imaged with excitation at
166 405nm.

167 *RT-PCR and RNA Sequencing*

168 Total RNA was extracted from mCCD_{cl1} cells and the clonal cell lines Ed1, Ed2 and Ed3 using TRIzol
169 (Ambion, Life Technologies). The concentration, purity and integrity of the RNA obtained were verified
170 using the 260-to-280-nm optical density ratio on a NanoDrop, and by running the RNA on a 1% agarose
171 gel to visualize ribosomal RNA 28S and 18S bands. cDNA was obtained using 500ng of RNA with a
172 High capacity RNA-to-cDNA Kit (Applied Biosystems). The primer sequences for each transcript were
173 obtained using PrimerBank (Table 1). The reactions were carried out in a Veriti 96-well Thermal Cycler
174 (Applied Bioscience) and the amplified PCR products separated by electrophoresis in a 2% agarose gel.

175 For RNA sequencing, mCCD_{cl1}, Ed1, Ed2 and Ed3 cells were cultured in T75 flasks for one week then
176 each line was passaged in three T25 flasks and cultured for one week before using Trizol to extract the
177 RNA. RNA was purified from genomic DNA using DNase Kit RNeasy Plus (Qiagen, USA). The process
178 gave 12 samples comprising of three replicates of each cell line. RNA purity and concentration were
179 determined as described above. Stranded total RNA libraries were prepared (Source Bioscience Plc
180 (Nottingham, UK)) according to the Illumina TruSeq Stranded mRNA sample preparation protocol and
181 validated on the Agilent BioAnalyser 2100. Illumina Paired-End multiplexed sequencing was undertaken
182 using the Illumina NextSeq sequencing platform. Read quality was checked using FastQC (2) and reads
183 were trimmed with Trimmomatic (3) yielding 20-58 million read pairs per sample. Reads were aligned
184 with HISAT2 (16) to the Ensembl mouse GRCm38 genome (mm10). Strandedness and read distribution
185 was assessed using RSeQC (35) and quasi-alignment using Salmon (24) for transcript quantification.

186 Estimated counts were adjusted for library size and transcript length using tximport (31). Matrices were
187 filtered and normalised using the trimmed Mean of M values method (26) and differential expression was
188 carried out using edgeR version 3.12.0 (25).

189 *Data analysis*

190 Data were analyzed using GraphPad Prism 7.0 (GraphPad Software) and statistical significance was
191 assessed using a Student paired t-test or one way ANOVA where appropriate. Data are expressed as mean
192 \pm SD, and n values refers to the number of repeats in an experiment. For each repeat, experimental
193 conditions were matched as closely as possible.

194 *Image analysis*

195 All the images were analyzed using ImageJ software (National Institutes of Health). Data were obtained
196 by measuring the mean grey value of the cell surfaces. The mean grey value corresponds to the mean
197 brightness level of the selected surface, and it was measured in 50 immuno-positive cells for each cell
198 passage ($n=4$) in the appropriate color channels, for a total of 200 immuno-positive cells per cell line. The
199 grey values attributed to background auto-fluorescence on the different channels were measured on a
200 control area (no cells) and subtracted from the grey values of the cells.

201

202 **Results**

203 *mCCD_{cl1} cells possess the functional characteristics of PCs but also express IC-related markers.*

204 Transepithelial electrophysiological measurements of parental mCCD_{cl1} cells revealed baseline I_{sc}
205 measurements of $-9.0 \pm 1.0 \mu A/cm^2$ ($n=4$), consistent with previous reports (11, 21). The application of
206 amiloride ($10 \mu M$, 10 min) to the apical bath inhibited I_{sc} by $82.1 \pm 8.2\%$, indicating that the basal current
207 can mostly, but not totally, be attributed to the transport of Na^+ via ENaC. The addition of aldosterone
208 ($3nM$, 3 h) increased I_{sc} by a factor of 3.8 ± 0.2 fold, to reach values of $-34.0 \pm 1.2 \mu A/cm^2$. I_{sc} , R_{te} and V_{te}
209 values are shown in Table 2. Cell polarization was observed using confocal microscopy on mCCD_{cl1}
210 stained with an anti- α -ENaC antibody (Figure 1e).

211 For characterization of the cell line, immunocytochemistry double-labelling analyses were performed on
212 mCCD_{cl1} cells using antibodies against Aqp2 and Cx30, Aqp2 and V-ATPase B1 (Figure 1a and 1b). or
213 α -ENaC and V-ATPase A1 (Figure 1c). The cells expressed the expected PC markers Aqp2 (green) and
214 α -ENaC (red). Unexpectedly, numerous cells also expressed the typical IC markers V-ATPase B1 and
215 Cx30 (Figure 1a and 1b). At higher magnification, the localisation of Cx30 and Aqp2 staining in mCCD_{cl1}

216 cells shows that while some cells only stain for PC or IC markers, many cells express markers of both
217 cells, suggesting an “intermediate” or transition cell type (Figure 1d). A similar phenotype was observed
218 with cells stained for V-ATPase B1 and Aqp2. Whilst 47% of total cells did not show any significant
219 staining, 42% of cells stained for both markers (dual-character/bi-potential), while cells staining for only
220 Aqp2 represented ~9% and cells with V-ATPase ~2%.

221 Immunocytochemistry using an anti-acetylated α -tubulin antibody showed that very few cells staining
222 positive for α -ENaC displayed primary cilia, and in those that did the cilia appeared short (Figure 2c). The
223 anti-acetylated α -tubulin antibody also stains the intracellular acetylated micro-tubular cytoskeleton,
224 considered more stable than its non-acetylated counterpart. Cells displaying strong V-ATPase A1 staining
225 showed a lack of staining for acetylated α -tubulin (Figure 2a). This observation was confirmed by the
226 quantification of mean grey value for both markers in 60 immuno-positive cells displaying a range of
227 different V-ATPase A1 staining intensities, with mean grey values $< 5\%$ considered low, and $>5\%$
228 considered high. A paired t-test applied to both populations (high or low V-ATPase A1) showed a
229 significant difference between the mean fluorescence intensities of V-ATPase A1 and acetylated α -
230 tubulin, and an inverse correlation between these two markers (Figure 2b). These data suggest that an
231 acetylated alpha-tubulin-positive cytoskeleton could be used as an additional marker for PCs as well as
232 cells possessing both PC and IC characteristics. The immunocytochemistry data led to the description of
233 four groups of cells, PC^+/IC^- , PC^-/IC^+ , PC^+/IC^+ , and PC^-/IC^- the characteristics of which are summarized
234 in Table 3.

235 *The bi-potential PC-IC cell phenotype is transmitted through a single mCCD_{cl1} cell to clonal cell lines.*

236 Dilution cloning of the mCCD_{cl1} (parental) cell line produced 8 clonal sublines derived from single cells,
237 among which the exemplary Ed1, Ed2 and Ed3 were selected for further studies based on their
238 morphology displayed at confluency, in particular their different average cell size. RT-PCR of Aqp2 and
239 Cx30 (Figure 3c) showed expression of both PC and IC markers in all three sublines.
240 Immunocytochemistry of V-ATPase B1 and Aqp2 (Figure 3a) in the three sublines showed the presence
241 of PC^+/IC^+ characteristics, as described above for the parental line. These PC^+/IC^+ cells are detectable in
242 different proportions when comparing each clone and the parental line (Figure 3b). PC^+/IC^+ cells
243 comprised $24.1 \pm 7.1\%$ of Ed1, $32.8 \pm 7.2\%$ of Ed2 and $45.5 \pm 5.7\%$ of Ed3 respectively. PC^-/IC^+ cells
244 were most represented in Ed2 with $5.7 \pm 1.9\%$. Ed3 is the closest to the parental line, with PC^+/IC^+ cells
245 representing $45.5 \pm 5.7\%$ of the total. In the parental and clonal sublines, the PC^+/IC^+ group makes up
246 more than 50% of the cells showing significant staining, showing that the capacity for the cells to possess
247 both IC and PC characteristics can be transmitted through a single cell of the mCCD_{cl1} parental line.

248 The data from the α -ENaC staining (Figure 3d and 3e) can be compared with the electrophysiological
249 measurements performed on the clonal sublines (Figure 4a-d and Table 2). Basal currents in Ed1 and Ed3
250 were 81.8 ± 7.0 and $88.4 \pm 3.1\%$ amiloride sensitive, respectively, indicating that, similar to the parental
251 line, their basal currents can principally be attributed to the transport of Na^+ via ENaC. Baseline I_{sc} in
252 Ed2, showed significantly lower ENaC expression levels (mean grey value at $2.0 \pm 0.4\%$ vs $7.2 \pm 0.9\%$
253 respectively) was negligible compared to the parental line ($-1.4 \pm 0.4 \mu\text{A}/\text{cm}^2$ v $-9.0 \pm 1.0 \mu\text{A}/\text{cm}^2$,
254 respectively). Ed1, consisting of $\sim 90\%$ of dual-staining cells, did not develop a cell layer as resistive as
255 the parental line, with a maximum R_{te} at day 10 of $0.8 \pm 0.1 \text{ k}\Omega \cdot \text{cm}^2$. V_{te} of Ed1 cells also remained lower
256 than for the parental line throughout the experiments, reaching a maximum of $-18.1 \pm 0.5 \text{ mV}$ on day 8,
257 compared to V_{te} of parental cells at day 8 of $-32.7 \pm 1.0 \text{ mV}$. These electrophysiological measurements
258 for Ed1 reflect the ENaC expression level, which at $3.5 \pm 0.4\%$ sits between Ed2 and mCCD_{c11}. Ed3 was
259 closer to the parental line in terms of ENaC immunostaining levels (mean grey value at $7.1 \pm 0.6\%$), and
260 developed strong R_{te} and V_{te} , significantly greater than the parental line. Baseline I_{sc} for mCCD_{c11}, Ed1
261 and Ed3 were similar, but the aldosterone responses of the sub-lines were significantly lower than the
262 parental line, with I_{sc} fold changes for baseline-to-aldosterone treatment at 3.8 ± 0.3 for the parental line,
263 and 2.1 ± 0.2 for Ed1 and Ed3 (Table 2). The responses to aldosterone and amiloride for Ed2 were
264 considered not relevant due to the negligible baseline I_{sc} . Electrophysiological measurements made from
265 clonal sublines and the parental line remained consistent throughout experiments (n=4), suggesting that at
266 a cell population level, the cells exhibit a stable phenotype.

267 *RNA Sequencing confirms important differences between parental line and sublines, and also expression*
268 *of IC-specific proteins.*

269 RNASeq was performed on RNA obtained from parental mCCD_{c11} cells and the Ed1, Ed2, and Ed3 (n=3).
270 Visualization of the data by principal component analysis shows the differences between the clonal lines
271 and the parental line. The first three principal components (PC1-3) represent 90% of the total variability
272 observed in the dataset (Figure 5a). PC1 corresponds to 59.8% of total variability, PC2 18.3% and PC3
273 11.9%. Whilst the repeats are grouped together, indicating the stability of expression between different
274 samples of the same cell line, the projection of the data on the three main principal components axis
275 shows heterogeneity of expression between the parental mCCD_{c11} cells and the three sublines. These
276 differences are easily visualized using a clustering dendrogram (Figure 5b), showing the variance between
277 the four cells lines by using the top 1000 genes with the most important variability between samples. The
278 extent of the difference between mCCD_{c11} and the three sublines can be interpreted by looking at the
279 height of the bars linking each sample. The data show that whilst the transcriptome was reproducible
280 between repeats, it differed significantly between the four lines and that Ed3 and Ed1 showed the greatest

281 similarity and difference, respectively, with mCCD_{cl1}. The top twenty transcripts showing the widest
282 differential expression between parental and clonal cell lines, highlighting the heterogenous nature of the
283 mCCD_{cl1} cell line are shown in Table 4.

284 As predicted from the immunocytochemistry data, the expression of genes associated with differentiated
285 ICs and PCs varied between the individual clones. Expression of the α -IC-specific sodium potassium
286 chloride co-transporter NKCC1 (SLC12A2), β -IC specific genes such as the sodium bicarbonate
287 exchanger NDCBE-3 (SLC4A8), the ATPase H⁺ transporter (Atp6v1b1), the potassium chloride co-
288 transporter KCC (SLC12A5) and the chloride channel CICK2 (CLCNKB), and the α/β -IC-specific
289 sulphate transporter SLC26A11 were observed in the parental cell line and all three sub-clones. PC-
290 specific genes such as the renal outer medullary potassium channel ROMK (Kcnj1), Kir4.1 (Kcnj10),
291 11 β HSD2 (HSD11B2), aquaporin 4 (AQP4), and sodium-potassium ATPase (ATP1A1) were also
292 detected in all four cell lines (Table 4 and data not shown).

293 The RNA-Seq data also reveals expression of a number of progenitor cell markers, including Pax2, p63,
294 CD24, CD133, Sca-1, and NfatC1, the latter previously ascribed to apoptosis-resistant renal progenitor
295 cells (19). The expression of specific collecting duct precursor cell markers in the RNA-Seq was
296 confirmed by staining the parental mCCD_{cl1} cells and the clonal lines for p63, which has been observed in
297 the ureteric bud, and Δ Np63 (7). All the cell lines show significant staining for both markers (Fig. 6a), but
298 with variable sub-cellular localization: p63 was either localized around the nucleus or, infrequently, more
299 widely throughout the cell. The p63 marker was found to co-localise with V-ATPase B1 (Fig. 6b).

300

301 **Discussion**

302 *mCCD_{cl1} cells display bi-potential characteristics*

303 At the population level our electrophysiological data show that mCCD_{cl1} cells exhibited the expected
304 transport characteristics of PCs, however the immunocytochemistry and RNA seq data suggest that
305 results from the functional data cannot be extrapolated to the behaviour of individual cells.

306 Electrophysiological measurements from the parental mCCD_{cl1} cell line gave results comparable to
307 previous studies, including a 3.8 ± 0.2 fold change in I_{sc} following treatment with physiological
308 concentration of aldosterone (33). This response was due to an increase in Na⁺ reabsorption via ENaC,
309 but the amiloride-insensitive I_{sc} demonstrates that a portion of the current is due to other electrogenic
310 transport. Indeed application of BaCl₂ inhibited part of the remaining current (data not shown), indicating
311 K⁺ secretion, likely via ROMK. Our data show that mCCD_{cl1} cells, widely used as representative of PCs

312 for electrophysiology studies, expressed significant levels of IC markers. It is therefore possible that H⁺
313 secretion via the apical V-ATPase in β -ICs may contribute to remainder of the amiloride-insensitive I_{sc}.

314 The presence of IC-specific transporter transcripts (for example the Na⁺/H⁺ exchanger (NHE3), NKCC1
315 (20), or H⁺/K⁺ ATPase (27)) suggests the possibility of IC-specific electroneutral ion transport. Such ion
316 transport mechanisms would not be detectable in our electrophysiology measurements. The
317 immunocytochemistry results showed that markers for both cell types were co-expressed in a significant
318 portion (~42%) of the cell population and that the cell line is not simply a mixed population of
319 differentiated PCs and ICs. The preponderance of cells expressing both PC and IC markers was also
320 evident for the clonal sub-lines, thereby reinforcing the conclusion that this is an intrinsic property of
321 mCCD_{cl1} cells.

322 The ability of mCCD_{cl1} cells to display differentiated characteristics of both PC and IC cells is
323 reminiscent of the bi-potential of cell lines such as HepaRG (23). In addition to expressing genes
324 characteristic of ICs and PCs our RNAseq data showed the expression of progenitor markers. The
325 mCCD_{cl1} cell line originated from a confluent primary culture of microdissected CCDs as a clone that
326 spontaneously continued to divide in culture. Its capacity for generating both IC- and PC-like cells
327 suggests that the immortalisation event(s) occurred in a bi-potential precursor cell resident in the CCD.
328 The fact that the cell line was isolated from the adult tissue raises important questions regarding the
329 potential for continuous physiological plasticity of the CCD *in vivo*. Further, the data provide evidence for
330 the inter-relationship between these two anatomically co-localised cell types, however the details of this
331 relationship cannot be determined from our present studies. Interestingly, the expression of p63 in
332 particular hints at the pluripotent nature of the mCCD_{cl1} cell line, and the potential for the line to be used
333 as a model for differentiation and determination studies.

334 *The immunocytochemistry results are consistent with the electrophysiological measurements*

335 The presence of PC⁺/IC⁺ cells was transmitted through single cells of the parental mCCD_{cl1} line to Ed1,
336 Ed2, and Ed3, although in different proportions. The comparison of electrophysiological and
337 immunocytochemistry data suggests that the composition of each cell line parallels their function. One
338 might expect that a higher proportion of ENaC-expressing cells would result in higher Na⁺ transport,
339 however the presence of dual-staining cells complicates the picture. The parental cell line and Ed1 have a
340 comparable proportion of cells expressing ENaC, but V-ATPase B1 is expressed in a greater number of
341 Ed1 cells correlating with lower R_{te} and V_{te} values than in the parental line. A similar relationship
342 between phenotype and function was observed for Ed2 and Ed3. The particularly small R_{te} measured for

343 Ed2 may be the result of a transitioning or undifferentiated state during which cells lose, or have not
344 established, features such as tight junctions.

345 *Transmission of both PC and IC characteristics to clonal sublines shows cell plasticity*

346 Plasticity of the mCCD_{cl1} cells was confirmed by the data obtained from the mCCD_{cl1} sub-lines following
347 single cell cloning. The nature of the PC⁺/IC⁺ cells could be described as bi-potential or displaying an
348 ‘immature’ phenotype characteristic of precursor cells. There is evidence for cells transitioning from α to
349 β -IC and from IC to PC *in vitro* (8, 34) but data on our clonal mCCD_{cl1} sublines suggests a substantial
350 degree of plasticity rather than uni-directional differentiation. The *in vivo* studies on Adam10 by Guo *et al*
351 (14) confirms the existence of factors influencing the fate and ratio of collecting duct IC and PC cells
352 through the Notch signalling pathway. They also showed that expression of Foxi1, which is important in
353 the differentiation of IC cells, was altered, supporting the case for the maintenance of collecting duct cell
354 plasticity *in vivo*. Ambiguous cell types (“hybrids”) were observed by Wu *et al.* (37), where *Dot1l*
355 deletion resulted in a ~15% rise in the number of ICs, seemingly derived from Aqp2⁺ cells. In the same
356 manner, ambiguous non- α non- β ICs have been observed and are speculated to be caught in a process of
357 transition between α and β (27).

358 The RNA sequencing of mCCD_{cl1} and the sublines confirmed the heterogenous characteristics of the
359 clones by showing clear differences in expression between the lines. Whilst RNA expression is a good
360 indicator of general transcriptional conditions in a cell population, it does not necessarily translate to
361 protein concentration or function, but provides important evidence for the heterogeneity of the mCCD_{cl1}
362 cell population. These data suggest that studies using mCCD_{cl1} cells should take account of the mixed
363 nature of their phenotype and the influence that cell line composition may have, particularly when
364 measuring the response of a population of cells as a whole, e.g. electrophysiological experiments. The
365 precise culture conditions may affect cell phenotype to a greater or lesser extent and may, in part, explain
366 variability between experiments.

367 *The clonal cell lines maintain their characteristics through passaging*

368 The argument that the mCCD_{cl1} cell line represents a precursor-like state is supported by the fact that the
369 individual sub-lines maintain a stable sub-line-specific distribution of cell types and electrophysiological
370 properties for a minimum of four passages. Whether, after prolonged passaging, the sub-lines would
371 revert to a common distribution of cell types and electrophysiology closer to that of the parental mCCD_{cl1}
372 cells, is unknown. The mechanism through which the sub-lines maintain their differences over passages is
373 unclear but may reflect epigenetic differences, for example methylation status. It is clear that
374 measurements of certain phenotypes, such as the electrophysiological characteristics reported here, are

375 based on the population of cells as a whole and yet the characteristics of individual cells making up the
376 populations vary widely both within, and between, independent sub-lines derived originally from single
377 cells. This raises interesting questions regarding the gene expression profile across a population of cells
378 *versus* individual cells, and whether neighbouring cells, and the local environment, influence that
379 expression.

380 It is impossible retrospectively to determine the nature of the single cells that gave rise to each sub-line,
381 and they may have originated from any of the PC⁺/IC⁻, PC⁻/IC⁺, PC⁺/IC⁺, or PC⁻/IC⁻ mCCD_{cl1} cells from
382 the parental population. However, following cloning they each gave rise to progeny that included all four
383 phenotypic groups. We can speculate that all three sub-lines may have arisen from PC⁺/IC⁺ cells and that
384 only these cells have the capacity to produce progeny of all four classes. From the present study there is
385 no evidence to prove that this is the case and it does not alter the conclusions that mCCD_{cl1} cells have bi-
386 potential, display a spectrum of phenotypes from IC-like cells to PC-like cells, and that this potential can
387 be transmitted vertically via a single cell. Prospective isolation of single cells of each class may enable
388 further insights into the potential for differentiation of mCCD_{cl1} cells, as would further investigation of
389 the Notch pathway.

390 *In vivo*, ICs and PCs cells are clearly distinct. However, collecting ducts *in vivo* are in a highly regulated
391 environment (6, 32), constantly under the influence of physiological factors that may be the key to
392 keeping cells in a fully differentiated, more stable state. In this work, the correlation between the
393 expression of V-ATPase A1 and the absence of acetylated α -tubulin could be used to identify cell types,
394 but also suggests that the cells are structurally distinct, and that acetylation could be an important factor
395 for the determination of CCD cell type.

396 Studies using gene knockout mice reported changes in the proportion of ICs and PCs or the existence of
397 “hybrid” cells (37). Genetic models of kidney disease such as the syndrome of apparent mineralocorticoid
398 excess (SAME) (18) may be informative for understanding the factors influencing collecting duct cell
399 plasticity, through the observation of the CCD cellular response to induced transport modifications, for
400 example the impaired Na⁺ transport by ENaC in SAME. Whilst the consequences of common kidney
401 diseases on sodium transport in the collecting duct are well documented, no specific data has been
402 reported regarding the relative number of PCs and ICs, even though the ratio can affect renal fluid
403 homeostasis. The observation of kidney tubules in real time under changing conditions such as drug
404 treatment, acidosis, or sodium intake, could be a useful tool for recording functionally relevant shift
405 between PCs and ICs *in vivo*.

406 *Using mCCD_{cl1} to develop kidney regeneration models*

407 Since mCCD_{cl1} cells have the capacity to form a resistive, polarized monolayer, their bi-potential
408 precursor nature also makes the cell line an excellent model for studying remodelling, especially
409 considering their expression of collecting duct precursor marker p63. Human adult kidney progenitor cells
410 have been shown to be useful in the treatment of acute renal failure (29) and the characteristics of
411 mCCD_{cl1} cells make them potential candidates for the establishment of *in vitro* 3D models of the CCD.

412 Recent publication of single cell RNA-seq analysis of microdissected collecting ducts revealed a small
413 fraction of cells, which clearly expressed both PC- and IC-specific transcripts (5). The potential for *in*
414 *vivo* plasticity and adaptation of the collecting duct to physiological challenge or disease etiology through
415 a shift in the PC/IC ratio may provide new leads for investigating factors that modify renal disease. As
416 well as being used as a model of principal cells for electrophysiology studies, the mCCD_{cl1} cell line has
417 the potential to contribute to our understanding of cellular interconversion in the CCD and the factors
418 influencing differentiation, renal injury and repair.

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555 **Figure legends**

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557 **Figure 1.** *mCCD_{cl1} cells express both PC and IC markers.*

558 (a) Immunostaining of mCCD_{cl1} cells cultured on filters using anti-Aqp2 (green) and anti-Cx30 (red)
559 antibodies as PC and IC markers respectively. Scale bar 20µm (b) Immunostaining of mCCD_{cl1} cells
560 using anti-Aqp2 (green) and anti-V-ATPase B1 (red) antibodies PC and IC markers respectively. Scale
561 bar 20 µm (c) Immunostaining of mCCD_{cl1} cells using anti-α-ENaC (red) and anti-V-ATPase A1 (green)
562 antibodies. Scale bar 20µm (d) Higher magnification of Aqp2 (green) and Cx30 (red) immunostaining of
563 mCCD_{cl1} cells. Scale bar 10µm. In all the merged pictures, DAPI staining of cell nuclei. (e) Confocal
564 image and orthogonal projections (right and bottom) of mCCD_{cl1} cultured on filter with α-ENaC
565 immunostaining in red over the greyscale autofluorescence of the cells. The orthogonal planes correspond
566 to cells along the crosshairs (red lines). White scale bar is 20µm.

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569 **Figure 2.** *Acetylated α-tubulin staining detects primary cilia, but also tubular cytoskeleton of PC-like*
570 *cells.*

571 (a) Immunostaining of mCCD_{cl1} using anti acetylated α-tubulin (green), anti-V-ATPase A1 (light blue)
572 and anti-α-ENaC (red) antibodies. DAPI staining of cell nuclei in the merged picture. The magnified area
573 highlights a region of interest where the inverse correlation between V-ATPase A1 and acetylated α-
574 tubulin, quantified in (c), is particularly notable. Scale bar 20µm. (b) Immunostaining of acetylated α-
575 tubulin and α-ENaC, focused on the apical membrane of mCCD_{cl1} cells to detect primary cilia, shown by
576 the white arrows. Scale bar 20µm. (c) Mean grey value of cells showing different expression levels of V-
577 ATPase A1 and acetylated α-tubulin. Left panel: paired t-test, orange for high levels of V-ATPase A1 ,
578 blue for low levels. Right panel: data ordered by increasing V-ATPase A1 mean grey value. Red dotted
579 line at 5% indicates the limit chosen to define high and low levels of V-ATPase A1.

580

581 **Figure 3.** *Both PC and IC phenotypes are transmitted to the clonal cell lines, in different proportions.*

582 (a) Representative images of mCCD_{cl1}, Ed1, Ed2, and Ed3 stained with anti-Aqp2 (green) and anti-V-
583 ATPase B1 (red) antibodies. DAPI staining of cell nuclei is shown in the merged pictures. Magnification
584 x40. Scale bar 20µm. (b) Quantification of the proportion of cells (%) staining for Aqp2 only, V-ATPase
585 B1 only, both, or neither in mCCD_{cl1}, Ed1, Ed2, and Ed3. (c) RT-PCR results of Aqp2 and Cx30 in the
586 mCCD_{cl1} parental (Par), and clonal lines Ed1, Ed2, and Ed3. C1, C2 are negative controls. (d)

587 Representative images of mCCD_{cl1}, Ed1, Ed2, and Ed3 cells stained with anti- α -ENaC (red), and DAPI
588 staining of cell nuclei. Magnification x40. Scale bar 20 μ m. (e) Mean grey value per cell line showing
589 different expression levels of α -ENaC in mCCD_{cl1}, Ed1, Ed2, and Ed3 cells.

590 **Figure 4.** *Parental mCCD_{cl1} and subline cells have different electrophysiological properties*

591 (a) Transepithelial voltage (V_{te}) measured across monolayers of mCCD_{cl1}, Ed1, Ed2, and Ed3 cells grown
592 on Snapwells filters, between day 3 and 10 after seeding. (b) Transepithelial resistance (R_{te}) measured
593 across monolayers of mCCD_{cl1}, Ed1, Ed2, and Ed3 cells. (c) I_{sc} was calculated using Ohm's law. Values
594 are shown as mean \pm SEM (n=4). (d) Effects on baseline I_{sc} of aldosterone (3nM) and amiloride (10 μ M,
595 apical bath) added at t=0 and t=120min respectively. Values are shown as mean \pm SEM (n=4).

596 **Figure 5.** *Transcriptomes of mCCD_{cl1}, Ed1, Ed2, and Ed3 are distinct.*

597 (a) Principal component analysis results displayed in a 3D matrix. The axes are the first three principal
598 components (PC1, PC2, and PC3), with the corresponding percentage of total variability they represent in
599 the dataset. Each geometric figure represents one sample, with the repeats (n=3) given the same shape. (b)
600 Clustering dendrogram analysis, based on the top 1000 genes with the most important variance between
601 the samples. The height of the bars is a measure of dissimilarity between samples. The repeats of the same
602 cell line are in the same shape.

603

604 **Figure 6.** *mCCD_{cl1}, Ed1, Ed2, and Ed3 express collecting duct progenitor markers p63 and Δ Np63*

605 (a) Representative images of mCCD_{cl1}, Ed1, Ed2 and Ed3 stained with anti-p63 (green) and anti- Δ Np3
606 (red) antibodies. DAPI staining in blue in the merged picture. Magnification x40. Scale bar 20 μ m. (b)
607 Representative images of mCCD_{cl1} stained with anti-V-ATPase B1 (red) and anti-p63 (green) antibodies.
608 DAPI staining in blue in the merged picture. Magnification x60. Scale bar 15 μ m.

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Tables

Table 1. Primers used for RT-PCR of Cx30 and Aqp2

	Gene	Genbank ID	Forward primer	Reverse primer	Product size, bp
Aquaporin 2	Aqp2	NC_000081.6	5'-atgtgggaactccggccata-3'	5'-acggcaatctggagcacag-3'	137
Connexin 30	Gjb6	NC_000080.6	5'-accagcataggaaggtgtg-3'	5'-tgcagagtgttcagacaaag-3'	119

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641 Table 2. Electrophysiological measurements for the parental mCCDcl1 cell line and the clonal sublines Ed1, Ed2
 642 and Ed3, and calculated I_{sc} fold change after aldosterone treatment and I_{sc} blocked from amiloride treatment.

		Baseline ± SD	Aldosterone 3h ± SD	Amiloride 10min ± SD	I _{sc} Fold change, aldosterone treatment ± SD	% of I _{sc} blocked by amiloride ± SD
mCCD_{cl1}	I _{sc} (μA/cm ²)	-9.0 ± 1.0	-34.0 ± 1.2	-1.6 ± 0.7	3.8 ± 0.3	82.1 ± 8.2
	R _{te} (kΩ·cm ²)	1.6 ± 0.1	1.0 ± 0.1	2.1 ± 0.1		
	V _{te} (mV)	-14.7 ± 1.5	-37.6 ± 0.8	-3.5 ± 1.6		
Ed1	I _{sc} (μA/cm ²)	-11.4 ± 0.9	-22.6 ± 0.7	-2.1 ± 0.8	2.1 ± 0.2	81.8 ± 7.0
	R _{te} (kΩ·cm ²)	0.8 ± 0.1	0.7 ± 0.0	1.0 ± 0.1		
	V _{te} (mV)	-8.7 ± 1.2	-16.0 ± 0.8	-2.1 ± 0.8		
Ed2	I _{sc} (μA/cm ²)	-1.4 ± 0.4	-4.1 ± 0.2	-0.9 ± 0.3	3.9 ± 1.2	36.4 ± 7.0
	R _{te} (kΩ·cm ²)	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.1		
	V _{te} (mV)	-1.2 ± 0.4	-3.4 ± 0.4	-0.9 ± 0.3		
Ed3	I _{sc} (μA/cm ²)	-12.8 ± 0.8	-27.1 ± 0.8	-1.4 ± 0.4	2.1 ± 0.2	88.4 ± 3.1
	R _{te} (kΩ·cm ²)	2.6 ± 0.2	-2.0 ± 0.1	4.1 ± 0.3		
	V _{te} (mV)	-33.1 ± 3.8	-54.0 ± 1.9	-6.3 ± 2.1		

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661 Table 3. Summary of the cell characteristics based on the immunocytochemistry data

	PC⁺/IC⁻	PC⁻/IC⁺	PC⁺/IC⁺	PC⁻/IC⁻
Aqp2 staining	yes	no	yes	no
V-ATPase B1 staining	no	yes	yes	no
Acetylated α-tubulin staining	high	low or absent	low or absent	low or absent

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673 Table 4. Top twenty differentially expressed transcripts between parental cell line and each clonal cell line, ordered
674 by decreasing fold change.

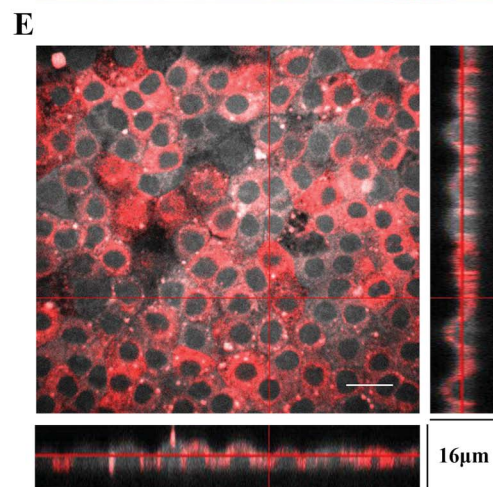
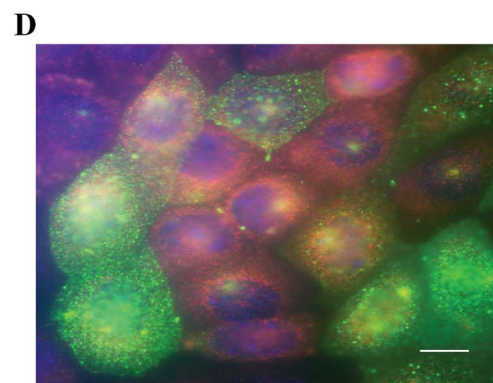
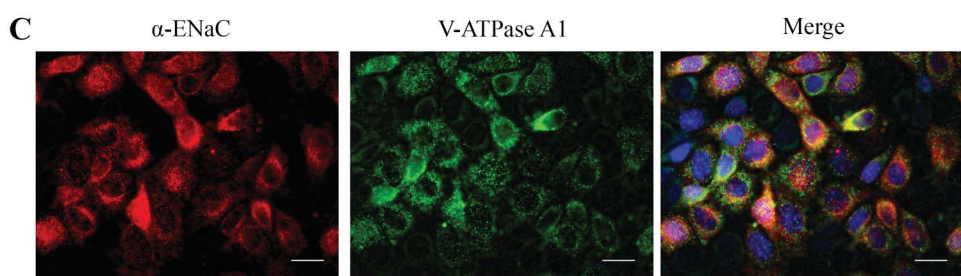
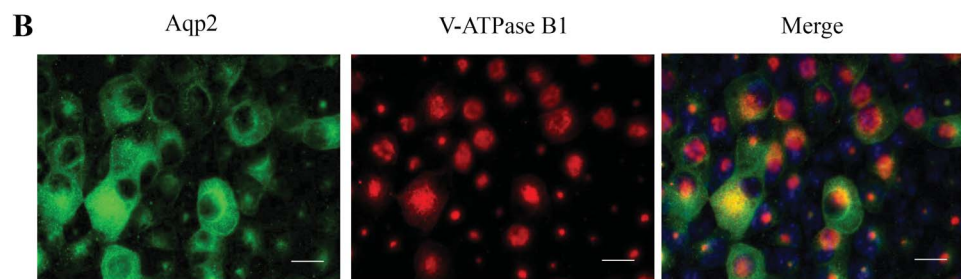
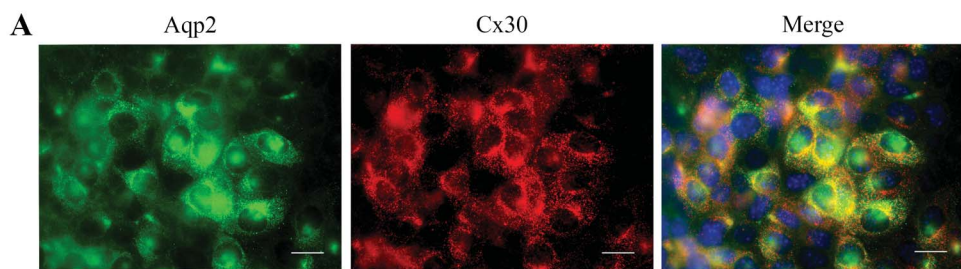
Parental vs Ed1				Parental vs Ed2			
Gene name	Ed1 (cpm)	Parental (cpm)	Fold change	Gene name	Ed2 (cpm)	Parental (cpm)	Fold change
<i>Gm20388</i>	0.12	94.13	784.42	<i>Pdlim1</i>	0.16	6.52	40.73
<i>Ldoc11</i>	0.12	6.79	56.58	<i>Crip1</i>	0.29	9.4	32.4
<i>Ap1m2</i>	0.37	15.47	41.82	<i>Kcnj10</i>	0.27	8.01	29.68
<i>Ndn</i>	0.19	5.5	29.48	<i>Rhcg</i>	1.99	56.87	28.53
<i>Gm13212</i>	0.61	14.7	24.23	<i>Sirpa</i>	0.14	3.67	26.21
<i>Egln3</i>	4.81	109.13	22.69	<i>Bsnd</i>	2	49.82	24.95
<i>Eya4</i>	0.14	2.31	16.5	<i>Nid2</i>	0.2	4.24	21.56
<i>Gm13157</i>	1.63	25.53	15.69	<i>Gm20388</i>	4.62	94.13	20.39
<i>Tmem22a</i>	0.21	3.22	15.56	<i>Arrdc4</i>	0.19	3.24	16.78
<i>Lhx1</i>	0.23	3.38	14.5	<i>Kcnj1</i>	1.63	26.93	16.55
<i>Igfbp5</i>	53.05	4.61	-11.5	<i>Tmem25c</i>	1.84	0.26	-7.09
<i>Ccl17</i>	8.09	0.7	-11.5	<i>Egr2</i>	11.26	1.26	-8.91
<i>Chst11</i>	3.43	0.29	-11.83	<i>Pga5</i>	3.78	0.41	-9.3
<i>Gm26822</i>	669.62	54.73	-12.24	<i>Nr4a3</i>	3.43	0.36	-9.45
<i>Gm15039</i>	7.28	0.44	-16.41	<i>Prss56</i>	1.42	0.15	-9.49
<i>Peg3</i>	684.3	38.41	-17.82	<i>Areg</i>	12.01	1.15	-10.44
<i>Nt5e</i>	16.32	0.8	-20.31	<i>Angpt2</i>	66.68	5.03	-13.25
<i>Mgp</i>	6.51	0.27	-23.82	<i>Gm9755</i>	4.47	0.28	-15.96
<i>Vgf</i>	12.77	0.53	-24.1	<i>Vgf</i>	17.4	0.53	-32.84
<i>Arvcf</i>	10.31	0.12	-85.94	<i>Arvcf</i>	9.96	0.12	-82.97

Parental vs Ed3

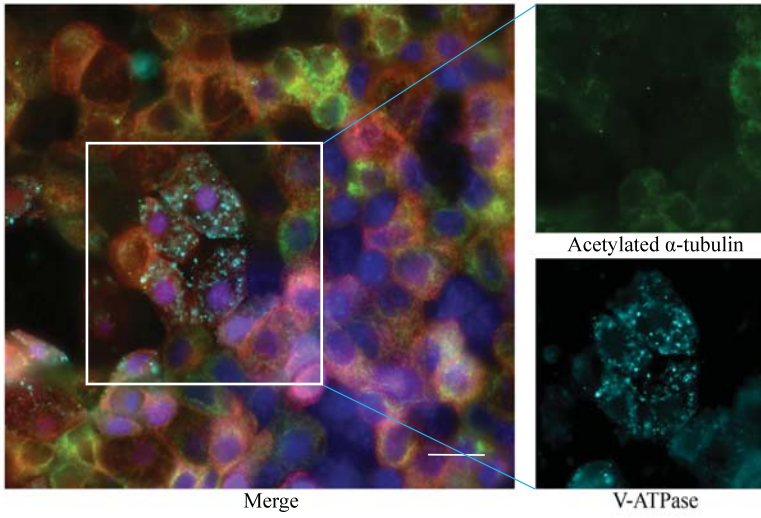
Gene name	Ed1 (cpm)	Parental (cpm)	Fold change
<i>Gm10443</i>	0.12	5.22	43.47
<i>Gm20388</i>	2.34	94.13	40.17
<i>Gm45140</i>	0.5	15.36	30.93
<i>Atp6v1b1</i>	0.56	10.89	19.57
<i>Kcnj1</i>	1.46	26.93	18.44
<i>Pcdh17</i>	2	34.27	17.17
<i>Naip6</i>	0.81	12.43	15.41
<i>Clcnkb</i>	0.22	3.1	13.87
<i>Eva1b</i>	0.18	2.28	12.45
<i>Fam84a</i>	0.12	1.43	11.92
<i>Loxl1</i>	2.83	0.49	-5.78
<i>Gm26778</i>	2.94	0.45	-6.59
<i>Mmp24</i>	7.31	1.1	-6.67
<i>Dmrtc1a</i>	1.51	0.2	-7.44
<i>Tmem254c</i>	1.94	0.26	-7.46
<i>Kctd12</i>	84.5	11.16	-7.57
<i>Gm11749</i>	1.7	0.18	-9.29

<i>Syt12</i>	60.2	6.37	-9.45
<i>Trps1</i>	6.51	0.69	-9.48
<i>Arvcf</i>	8.15	0.12	-67.89

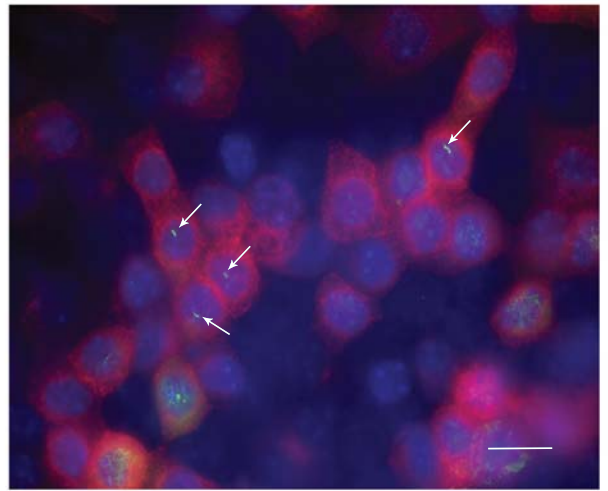
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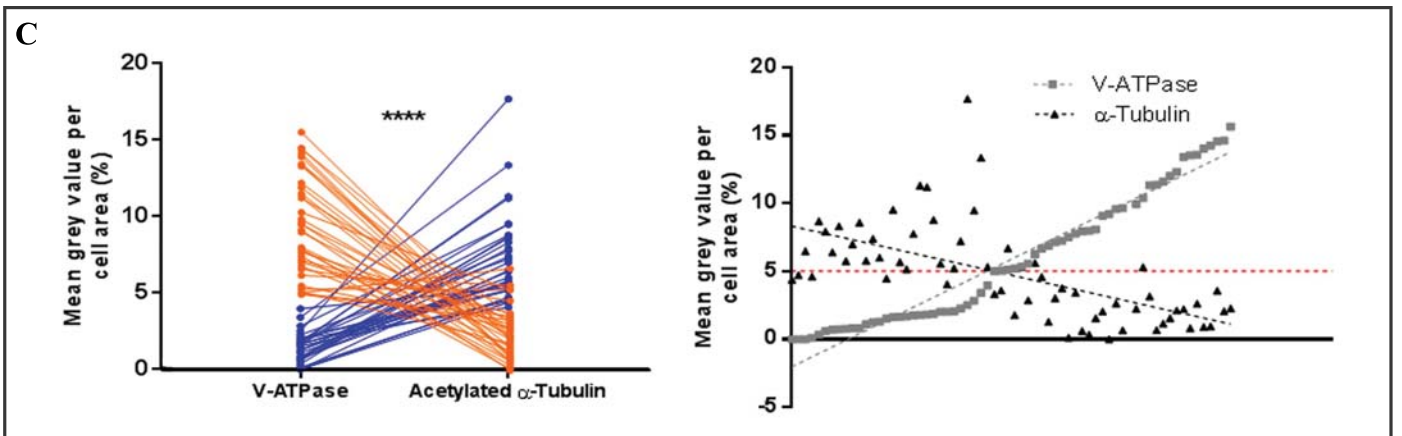
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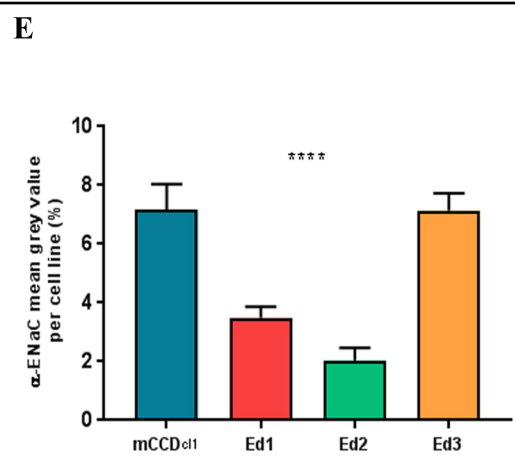
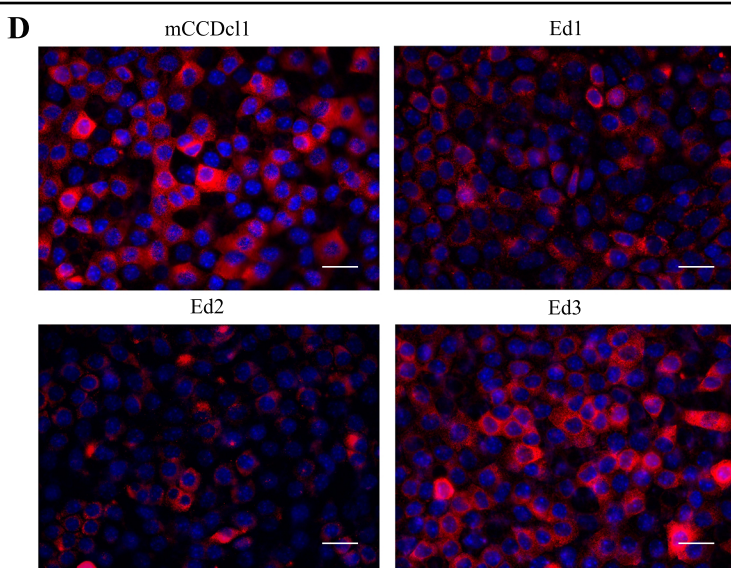
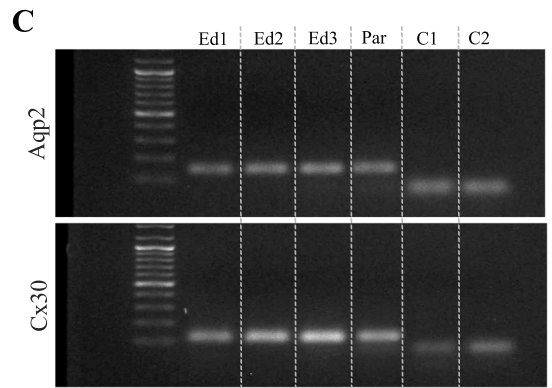
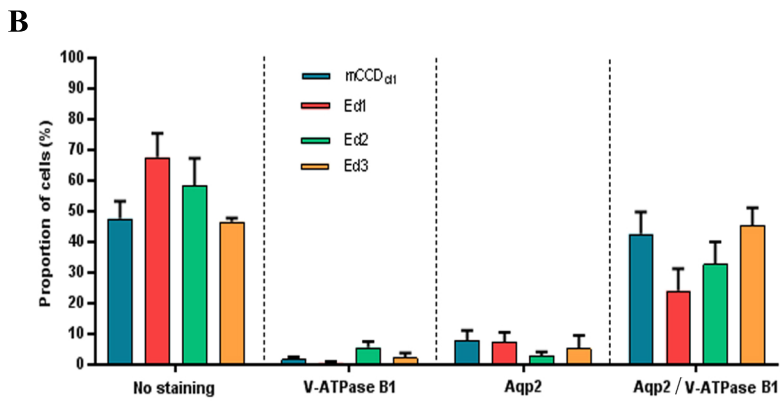
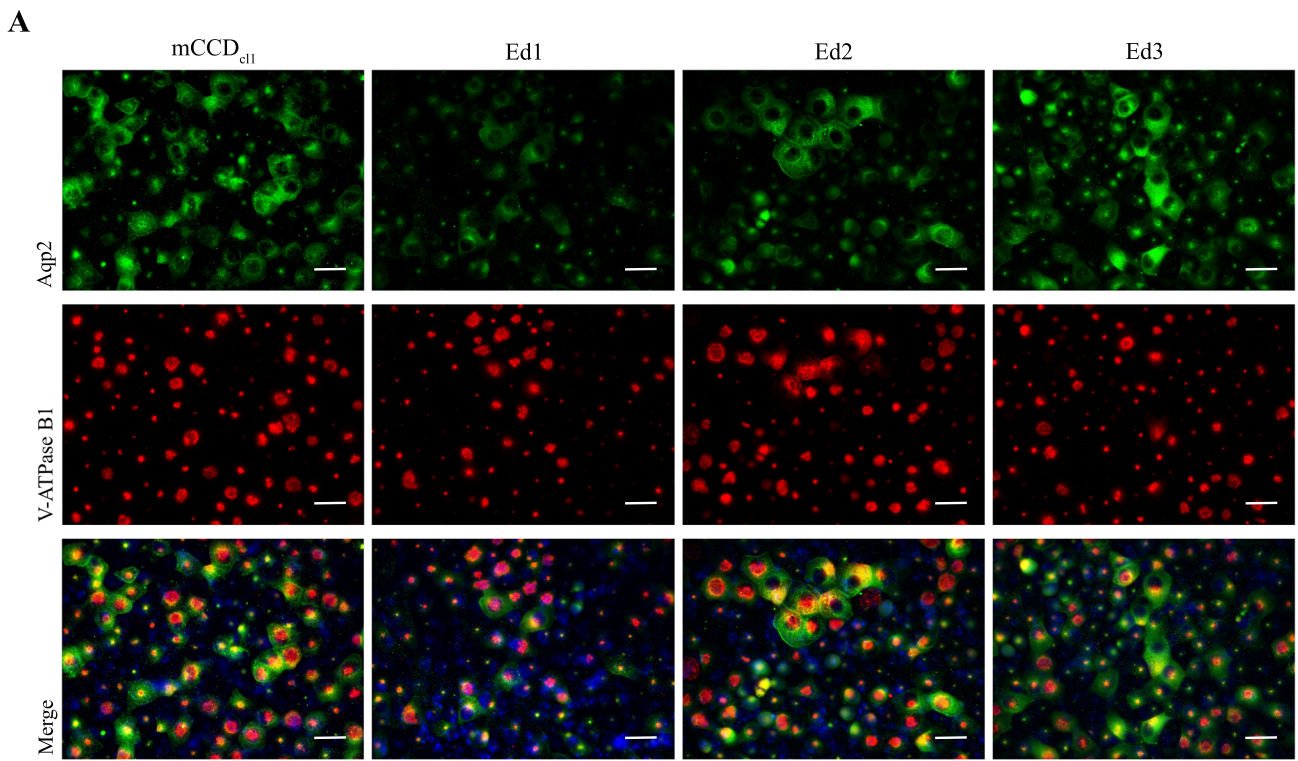


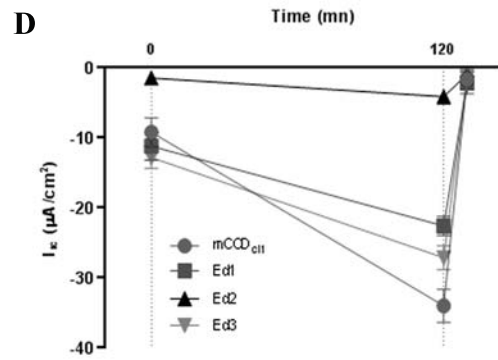
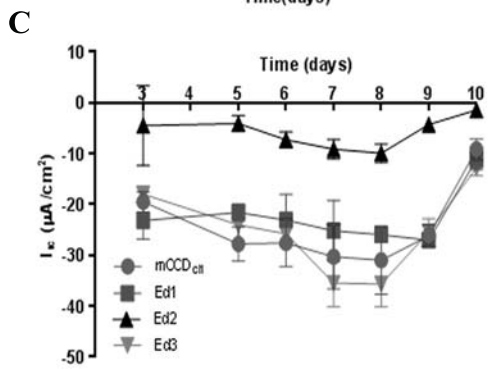
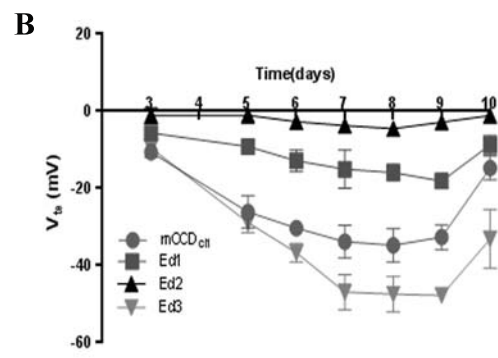
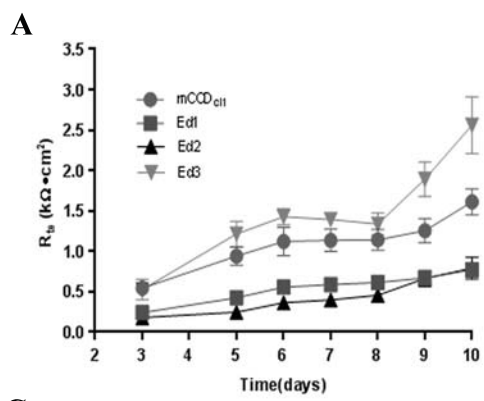
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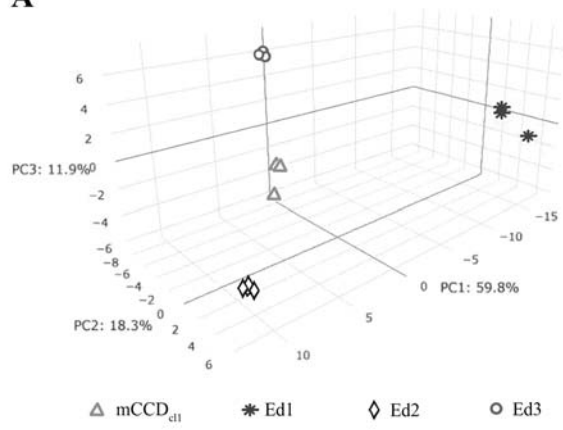


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A**B**