SUPPLEMENTARY MATERIAL

OCRL Deficiency Impairs Endolysosomal Function in a Humanized Mouse Model for Lowe Syndrome and Dent Disease

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MATERIALS AND METHODS

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Cell profiler quantifications. The pipeline "Speckle counting" derived from the open source Cell Profiler image analysis software was used to identify dots ($PI(4,5)P_2$ or PI3P or BSA or Dextran or LAMP1 or MPR-positive structures) surrounding nuclei and to perform per-nuclei aggregate measurements (eg. the number of dots/nucleus), as described by Carpenter et al. (2006) and De Leo et al. (2016). Briefly, the images were converted to greyscale using the module "ColorToGray". The "IdentifyPrimaryObjects" module was used to identify nuclei and dots while cells were identified by using the module "IdentifySecondaryObjects". "MaskObject" and "RelateObject" modules were used to establish a parent-child relationship between the cells and the dots, identified as masked objects, and to calculate the average of dots per cell. The module "MeasureObjectIntensityDistribution" was used to score the fluorescence intensity of MPR and TfR positive structures contained into perinuclear region (area defined by the first 10 bin) and peripheral region (area defined by the last 10 bin) of the cells. Briefly, the software generates 20 concentrically bin around the nucleus of each cell and calculates the intensity distribution for each bin. The module "MeasureObjectSizeShape" was used to score the vesicle size of LAMP1. The pipeline "Cell/particle counting, and scoring the percentage of stained objects" was used to score the percentage of $EEA1^+$ structures colocalizing with $PI(4,5)P_2$ or F-actin, the percentage of $LRP2^+$ structures colocalizing with F-actin, EEA1 or TfR and the percentage of $LAMP1^+$ structures colocalizing with $PI(4,5)P_2$, DQ-BSA or PepA. Briefly, the images were converted to greyscale using the module "ColorToGray". Then the "ObjectIdentification" module was used to identify the nuclei and the aforementioned stained structures/vesicles. The "RelateObject" module was used to establish a parent-child relationship between the stained objects. In this case, a "parent" object is one that touches, overlaps or encloses a "child" object. Objects1 that touch or overlap with an Object2 are considered to be colocalized and will be assigned as a parent to a corresponding child. The "ClassifyObjects" and "FilterObjects" modules were used to

categorize structures that were either colocalizing or not-colocalizing, and to calculate the percentage of colocalizing structures on the total amount per image. Fluorescence intensity of LRP2 was measured from a Z stack of 55 serial images acquired, according to Nyquist theorem, every 0.13 µm to maximize the spatial information. The average fluorescence intensity of LRP2 was calculated every 0.5 µm of the Z-stacks projection by using ImageJ software. Qualitative analysis of F-actin dynamics was performed by maximal projection of Z-stack images acquired throughout the entire volume of the cells.

Behavioral tests. ***Open field large arena test.*** The large open-field arena is circular with a diameter of 150 cm, a slightly roughened white plastic floor, and 35cm high smooth sidewalls made of white polypropylene. Illumination is performed by indirect diffuse room light (4 40W bulbs, 12 lux). Each subject is released near the wall and observed for 10 min on two subsequent days. Movements are tracked using Noldus EthoVision. Between subjects, the arena is thoroughly cleaned with water and dried. ***Water-maze cue navigation.*** The water maze is constituted by a round white poly-propylene pool with a diameter of 150 cm with 68 cm high walls. It is filled with water (24-26° C, depth 15 cm) which is rendered opaque by addition of 1l of milk (UHT whole milk 3.5% fat, Coop, Switzerland). The white quadratic goal platform (14x14 cm) is made of metallic wire mesh and painted white. It is hidden 0.5 cm below the water surface in the center of one of the 4 quadrants, approximately 30 cm from the side wall. Salient extra-maze cues made of black cardboard are placed on the walls of the testing room. Computer and experimenter are hidden behind a curtain. Illumination is performed by indirect diffuse room light (4 40W bulbs, 12 lux at the center of the pool). During the test animals are trained for 2 days with the goal platform marked by a salient cue and placed in a different quadrant for every trial. All subjects are trained on the same sequence of goal positions and released from the same start points. ***Three-chambers sociability test.*** The 3-chamber cage is a polycarbonate type III cage (20.5 cm high, 58 × 40

cm top, 55 × 37.5 cm bottom, Techniplast, 2000P, Buguggiate, Italy). Two dividers provided with gates regulate the access of the test mouse from the middle to the lateral chambers. One unfamiliar stimulus mouse is placed inside a metallic cylinder in one of the two lateral chambers. The cylinder allows visual and olfactory contact between the test mouse and the stimulus mouse, reducing the possibility of physical contact. In the other lateral empty chamber, an empty cylinder is placed giving then the test mouse the choice to spend time in the chamber with the novel object (the cylinder) plus the unfamiliar mouse (social stimulus) and the chamber containing only the empty cylinder. This test runs for 10 minutes after which the stimulus mouse is considered to be familiar to the test mouse. The time spent by the test mouse in the lateral chambers is noted.

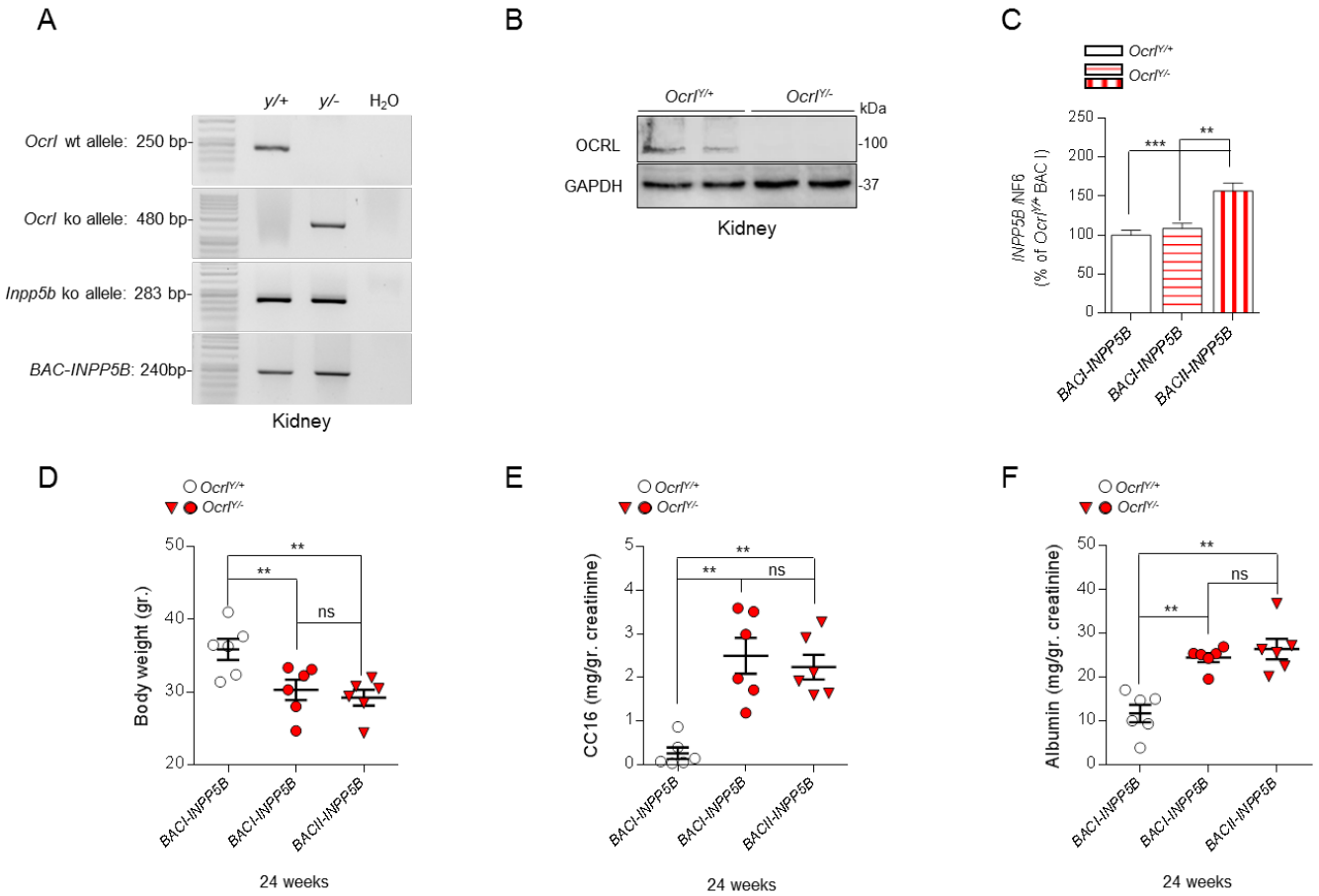
IntelliCage apparatus and mouse preparation. The IntelliCage apparatus (TSE Systems, Bad Homburg, Germany) is placed in a polycarbonate cage (20.5 cm high, 58 × 40 cm top, 55 × 37.5 cm bottom, Techniplast, 2000P, Buguggiate, Italy) and accommodates up to 16 mice. Its aluminum top contains a freely accessible food rack filled with standard mouse chow (Standard 3430, Kliba Nafag, Kaiseraugst, Switzerland). The floor is covered with woodchip bedding and provides 4 central red shelters (Techniplast, Buguggiate, Italy). Four triangular conditioning chambers (15×15×21 cm) are fitted in the cage corners and provide room for one mouse at a time. Each chamber contains two drinking bottles, accessible via two round openings that can be opened and closed with motorized doors. Mice were tagged with RFID-transponders, subcutaneously implanted under isoflurane anesthesia, which can be read by a circular RIFD antenna located at the entrance of each chamber. The duration of their visit is determined by both the antenna reading and a temperature sensor that detects the presence of the animal inside the corner. During a visit, number and duration of individual nosepokes at each door are recorded using IR-beam sensors. Licking episodes at each bottle are monitored using lickometers (duration of the episode, number of licks, total contact time). IntelliCages

have individual controllers and are connected to a central PC running the software that permits to design and control experiments remotely, as well as to analyze the recorded data (IntelliCage Plus, TSE Systems, Bad Homburg, Germany). After RIFD implantation, mice are allowed to recover for one week in groups of 8-16 mice in standard Type III cages with water and food ad libitum. The same grouping is maintained when the mice are later transferred to IntelliCages. During all adaptation phases and tasks in IntelliCage, mice are fed ad libitum. Ambient lights are on 20:00-08:00. Room temperature is kept constant at 23°C.

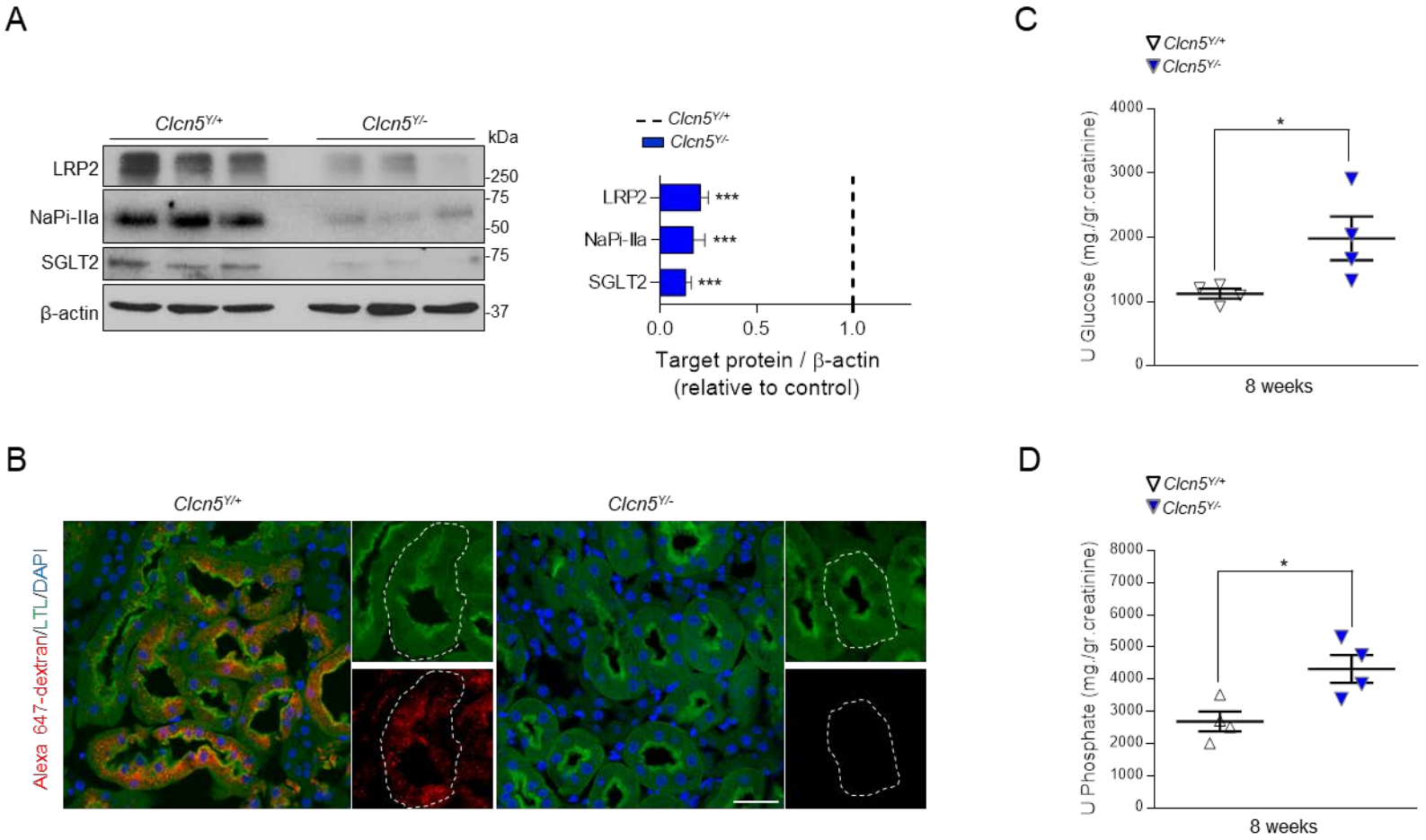
Additional References:

Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I.H., Friman, O., Guertin, D.A., Chang, J.H., Lindquist, R.A., Moffat, J. et al. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol*., **7**, R100.

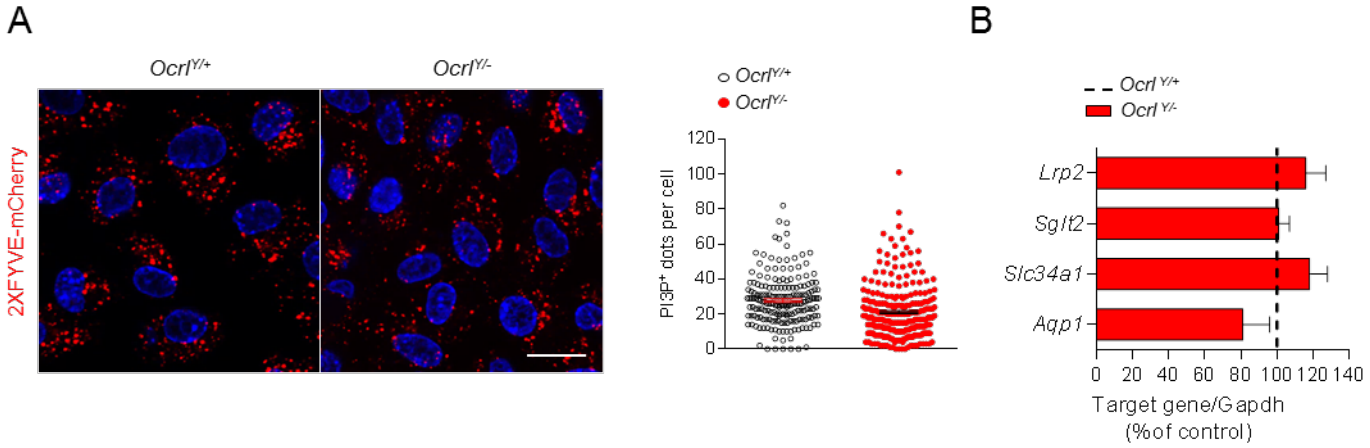
De Leo, M.G., Staiano, L., Vicinanza, M., Luciani, A., Carissimo, A., Mutarelli, M., Di Campli, A., Polishchuk, E., Di Tullio, G., Morra, V. et al. (2016) Autophagosome-lysosome fusion triggers a lysosomal response mediated by TLR9 and controlled by OCRL. *Nat. Cell. Biol*., **18**, 839-850.



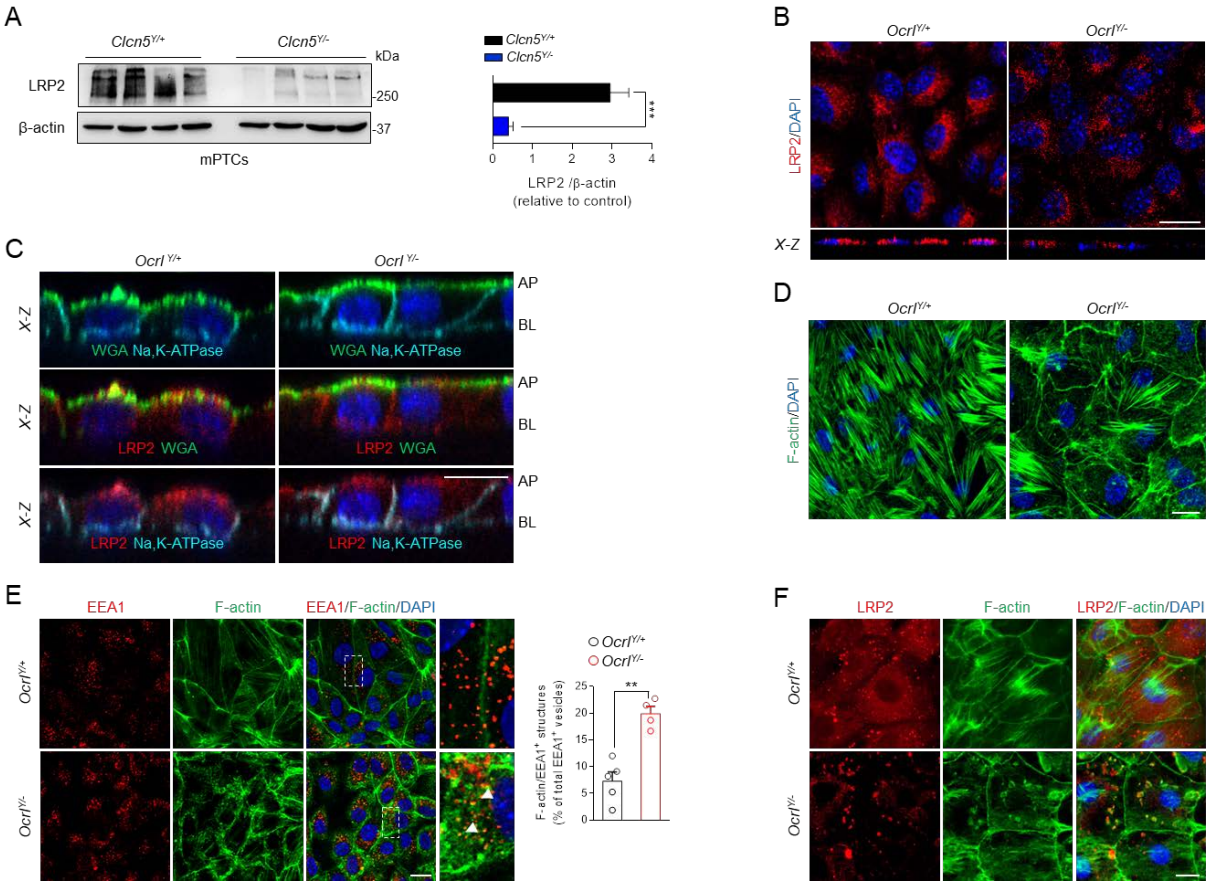
Supplementary Figure 1. **Genotype confirmation and analyses of the kidney phenotype in *Ocrl*$^{Y/-}$ mice harboring different levels of human *BAC-INPP5B* expression**. (**A**) *Ocrl*, *Inpp5b* and *BAC-INPP5B* genomic DNA analyses by PCR and agarose gel electrophoresis isolated from kidney biopsies derived from *Ocrl* mice. (**B**) Western blotting of OCRL protein level in whole kidney lysates from *Ocrl* mice. GAPDH was blotted as loading control. (**C**) *BAC-INPP5B* mRNA expression in kidneys derived from *Ocrl* mice was analyzed by quantitative RT-PCR. Gene target expression was normalized on 6 housekeeping genes and expressed as relative to *Ocrl*$^{Y/+}$ *BACI-INPP5B* (n=5 mice per condition). (**D**) Dot plot representing the measurement of the body weight, (**E**) Clara cell secretory protein (CC16) and (**F**) albumin urinary excretion in *Ocrl* mice harboring different level of *BAC-INPP5B* expression as shown in **C**. Each dot represents one mouse (n=6 mice per group). All the analyses in **D**, **E** and **F** were performed on mice matched for age (24 weeks) and gender (male). All the urine parameters were normalized to urinary creatinine concentration. Plotted data represent mean ± SEM. Two-tailed unpaired Student's t-test, **P < 0.01 relative to *Ocrl*$^{Y/+}$ or *Ocrl*$^{Y/-}$ *BACI-INPP5B* mice. ns: not significant.



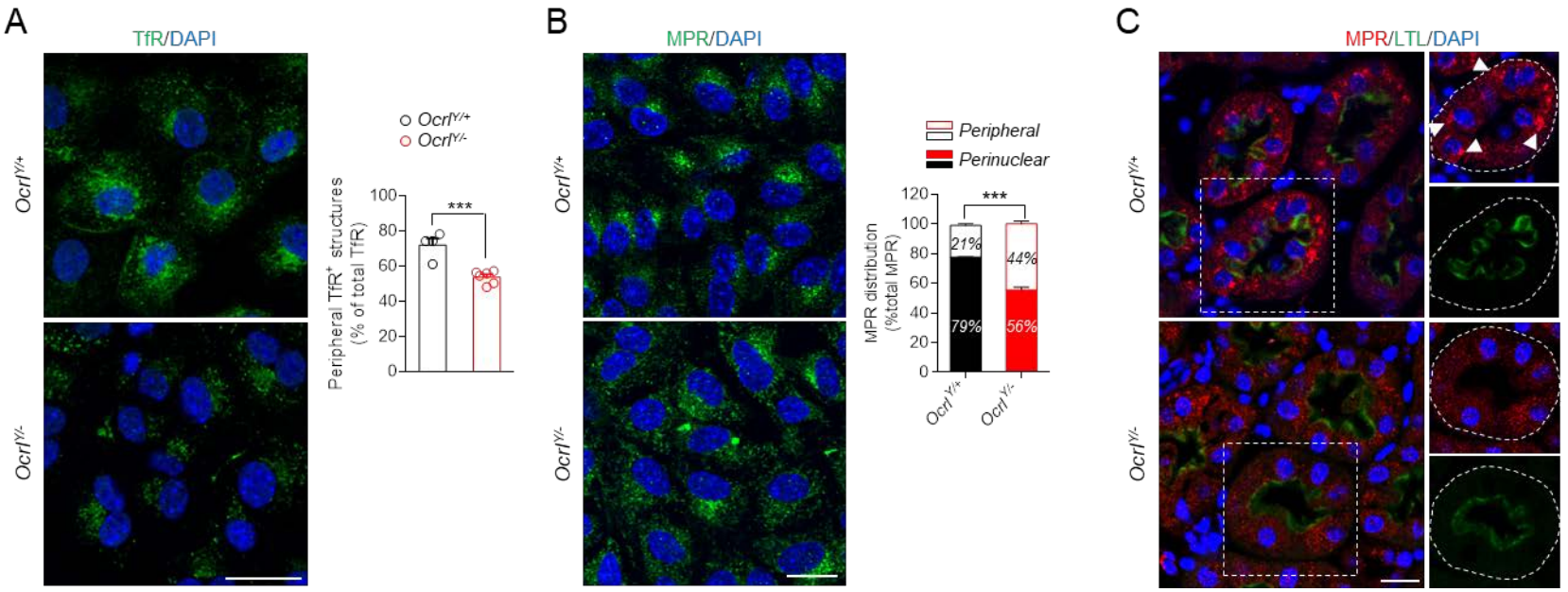
Supplementary Figure 2. **Proximal tubule dysfunction in *Clcn5*$^{Y/-}$ mice**. (**A**) Western blotting and densitometry analysis of LRP2, NaPi-IIa and SGLT2 protein levels in whole kidney lysates from *Clcn5* mice. β-actin was used as loading control. (n= 3 mice per group). (**B**) Representative confocal micrographs showing Alexa 647–labeled dextran uptake (red, 6 mg/kg B.W) in LTL^{+} (*Lotus Tetragonolobus Lectin*, green) proximal tubules of *Clcn5* mouse model after 30 minutes from tail vein injections. Nuclei counterstained with DAPI (blue). Scale bar 25μm. (**C**) Dot plot representing the measurement of glucose and (**D**) phosphate urinary excretion in 8 weeks old *Clcn5* mice. Each dot represents one mouse (n=4 mice per group). All the urine parameters were normalized to urinary creatinine concentration. Plotted data represent mean ± SEM. Two-tailed unpaired Student's t-test, *P < 0.05, ***P < 0.001 relative to *Clcn5*$^{Y/+}$ mice.



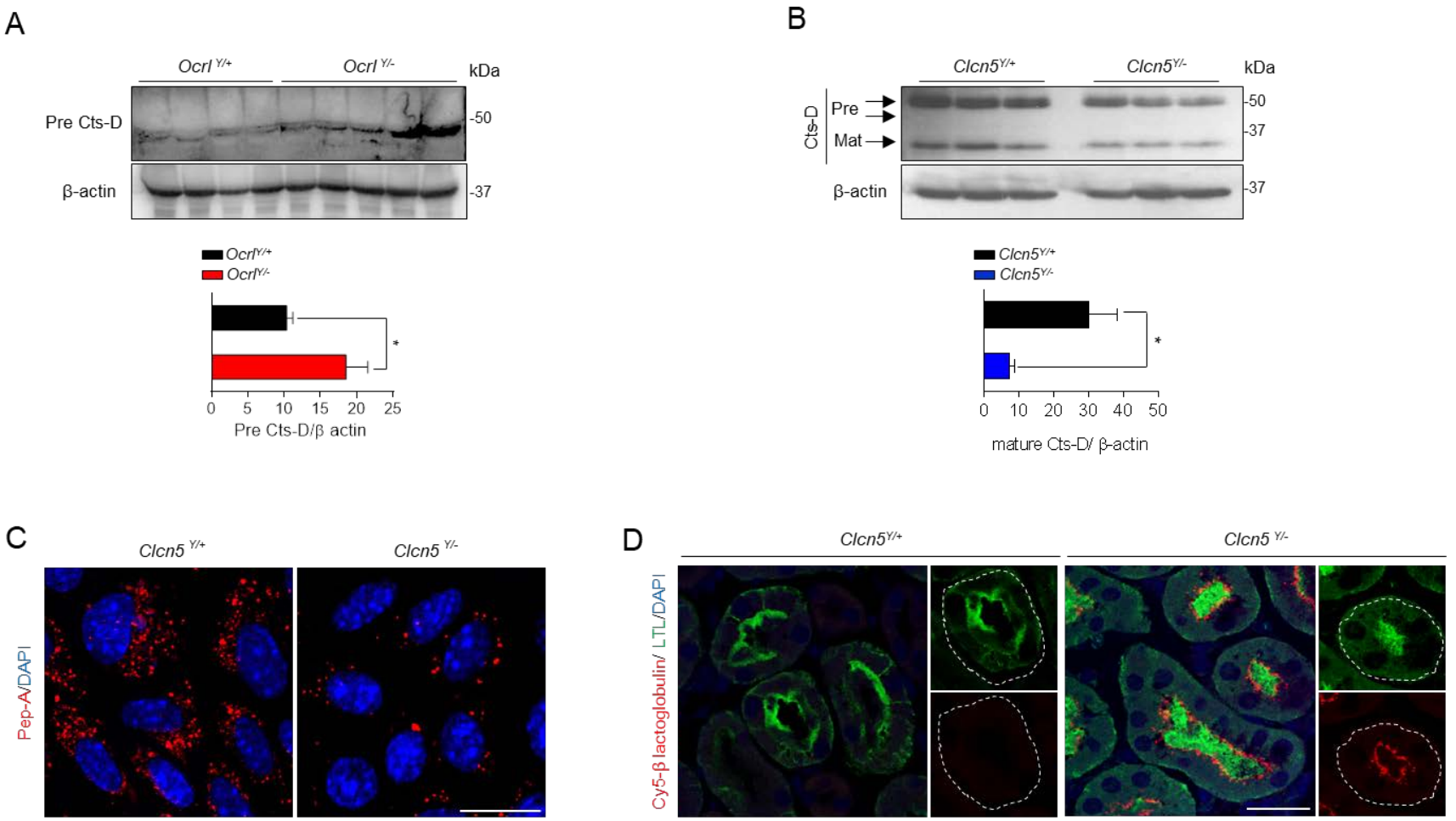
Supplementary Figure 3. *Ocrl*$^{Y/-}$ mPTCs exhibit no changes in PI3P (Phosphatidylinositol 3 phosphate) levels and no transcriptional alterations of proximal tubule receptors and channels. (**A**) Representative confocal micrographs and quantification of the number of PI3P^{+} structures stained by 2XFYVE-mcherry domain (red) in *Ocrl* mPTCs (n≈ 200-250 cells pooled from three *Ocrl* kidneys per group; each point representing the number of PI3P^{+} vesicles in a cell). Nuclei counterstained with DAPI (blue). Scale bar 15µm. **(B)** The mRNA mPTCs levels of *Lrp2*, *Sglt2*, *Slc34a1* and *Aqp1* were analyzed by real-time qPCR. Genes target expression normalized to *Gapdh* and relative to *Ocrl*$^{Y/+}$ (n = 3 mice per group). Plotted data represent mean ± SEM.



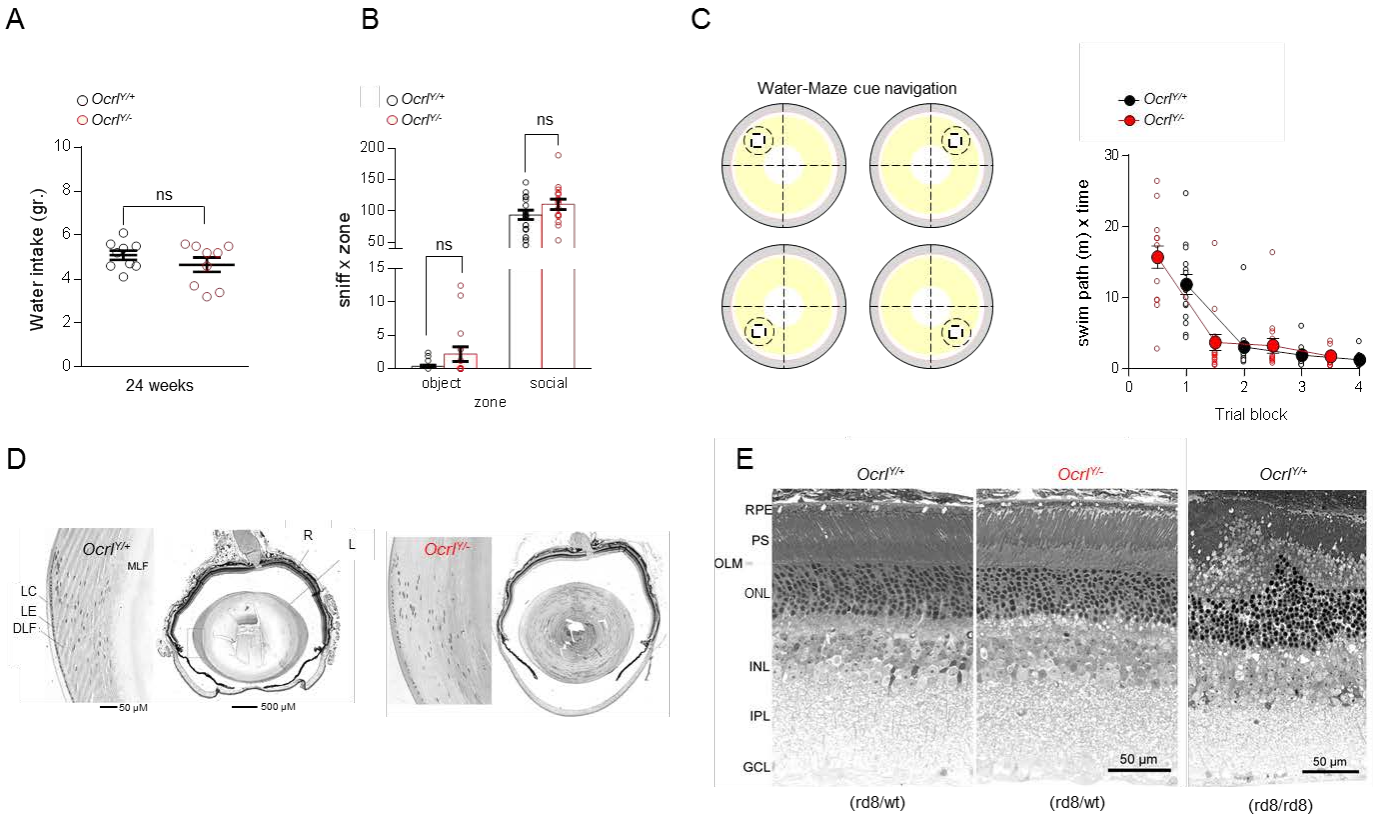
Supplementary Figure 4. LRP2 dynamics in Dent disease. *(*A) Western blot and densitometry analysis of LRP2 in whole *Clcn5* mPTCs lysates. β-actin was used as loading control (n= 4 mice per group). (**B**) Representative confocal micrographs and X–Z side view of *Ocrl* mPTCs immunostained with anti-LRP2 (red). (**C**) Representative confocal micrographs of X–Z side view of a z-stack performed on *Ocrl* mPTCs immunostained with WGA (green) and Na,K-ATPase (cyan) in the upper panels, WGA (green) and LRP2 (red) in the middle panels and Na,K-ATPase (cyan) and LRP2 (red) in the lower panels. (**D**) Maximum intensity projection of a confocal Z-stacks series representing *Ocrl* mPTCs immunostained with Alexa-Fluor-488-phalloidin (green, F-actin). (**E**) Representative confocal micrographs of *Ocrl* mPTCs immunostained with anti-EEA1 (red, early endosomes) and Alexa-Fluor-488-phalloidin (green, F-actin). Quantification of F-actin/EEA1^{+} structures (in percentage of total EEA1^{+} vesicles, n=5 *Ocrl*$^{Y/+}$; n=4 *Ocrl*$^{Y/-}$ randomly selected fields per condition, each containing ≈ 10-15 cells). Insets: high magnification of F-actin/EEA1^{+} structures. (**F**) High magnification confocal micrographs of *Ocrl* mPTCs immunostained with anti-LRP2 (red) and Alexa-Fluor-488-phalloidin (green, F-actin). Nuclei counterstained with DAPI (blue) in **B**-**F**. Scale bars in **C** 10µm**,** in **B**, **D**, **E** and **F** 15µm. Plotted data represent mean ± SEM. Two-tailed unpaired Student's t-test, **P < 0.01, ***P < 0.001 relative to *Clcn5* $^{Y/+}$ or *Ocrl* $^{Y/+}$ mPTCs.



Supplementary Figure 5. Impaired trafficking of MPR and TfR cellular receptors in *Ocrl*$^{Y/-}$ mPTCs. (**A**) Representative confocal micrographs and quantification of TfR^{+} structures distribution (green) in *Ocrl* mPTCs (in percentage of the total TfR^{+} vesicles, n=4 *Ocrl*$^{Y/+}$; n=6 *Ocrl*$^{Y/-}$ randomly selected fields per condition, each containing ≈20 cells).Two-tailed unpaired Student's t-test, ***P < 0.001 relative to *Ocrl*$^{Y/+}$ mPTCs. (**B**) Representative confocal micrographs and quantification of distribution of MPR^{+} structures (green) in *Ocrl* mPTCs (perinuclear vs peripheral in percentage of the total MPR^{+} vesicles, n=3 randomly selected fields per condition, each containing ≈40-50 cells). Plotted data represent mean ± SEM. Two-tailed unpaired Student's t-test, Peripheral/perinuclear MPR^{+} vesicles ***P < 0.001 relative to *Ocrl*$^{Y/+}$ mPTCs. (**C**) Representative confocal micrographs showing MPR^{+} structures (red) in LTL^{+} (*Lotus Tetragonolobus Lectin*, green) proximal tubules of *Ocrl* mouse kidneys. Plotted data represent mean ± SEM. Nuclei counterstained with DAPI (blue). Scales bars in **A** 20µm **B** and **C** 15µm.



Supplementary Figure 6. Altered lysosomal activity in Dent disease. (**A-B**)Western blotting and densitometry analysis of Cathepsin D (Cts-D) protein levels in (**A**) plasma samples from *Ocrl* mice (n= 4 *Ocrl*$^{Y/+}$ and n= 5 *Ocrl*$^{Y/-}$ mice) and in (**B**) *Clcn5* mPTCs (n= 3 mice per group). β-actin was used as loading control. Plotted data represent mean ± SEM. Two-tailed unpaired Student's t-test, *P < 0.05 relative to *Ocrl*$^{Y/+}$ plasma or *Clcn5*$^{Y/+}$ mPTCs. (**C**) Confocal micrographs showing *Clcn5* mPTCs immunostained with Bodipy-FL-PepA (1 μM, for 1 h at 37 °C, green). (**D**) Representative confocal micrographs showing Cy5 labeled β-lactoglobulin (red, 1mg/kg B.W.) after 120 minutes from tail vein injections in LTL^{+} proximal tubules from *Clcn5* mouse kidneys. Nuclei counterstained with DAPI (blue) in **C** and **D**. Scale bars 15μm in **C** and 25μm in **D.**



Supplementary Figure 7. **No impairment of social behavior neither defective ocular morphology is observed in *Ocrl*$^{Y/-}$ mice.** (**A**) Dot plot representing the measurement of water intake, over 12 hours, in 24 weeks old *Ocrl* male mice. Each dot represents one mouse. (**B**) Dotted bar graph representing the number of nose contact time with the social (cage with mouse) and object (empty cage) stimulus during the 3 chambers sociability test. Plotted data represent mean ± SEM. Each dot of the graphs represents one mouse. (**C**) Schematic representation of the water maze cue navigation test (left panel): animals are trained in the water maze to reach a platform marked by a salient cue and placed in a different quadrant for every trial. Dot plot (right panel) representing the swim path as function of 3 trial blocks. Each dot of the graphs represents one mouse. (**D**) Light microscopy representative pictures of high magnification hematoxylin-eosin stained lens (left panels, scale bars 50µm) and midsagittal sections of whole eyes (right panels, scale bars 500µm) derived from *Ocrl* mice. LC (lens capsule), LE (lens epithelium), DFL (differentiating lens fiber), MFL (mature lens fiber), L (lens) and R (retina). (**E**) Toluidine blue stained retina derived from *Ocrl* mice in presence or absence of the spontaneous rd8 (retinal degeneration 8) mutation in *Crb1* gene (right panel, rd8/rd8). RPE (retinal pigment epithelium), PS (photoreceptor segments), OLM (outer limiting membrane), ONL (outer nuclear layer), INL (inner nuclear layer), IPL (inner plexiform layer), GCL (ganglion cell layer); Scale bars 50µm. ns: not significant.

Table S1: Primer pairs for gene expression analysis.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	PCR products (bps)	Efficiency
Gapdh	TGCACCACCAACTGCTTAGC	GGATGCAGGGATGATGTTCT	176 bp	1.04 ± 0.03
Actb	TGCCCATCTATGAGGGCTAC	CCCGTTCAGTCAGGATCTTC	102 bp	1.03 ± 0.04
Hprt1	ACATTGTGGCCCTCTGTGTG	TTATGTCCCCCGTTGACTGA	162 bp	0.99 ± 0.01
Ppiase	CGTCTCCTTCGAGCTGTTTG	CCACCCTGGCACATGAATC	139 bp	1.02 ± 0.02
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151 bp	0.98±0.02
36B4	CTTCATTGTGGGAGCAGACA	TTCTCCAGAGCTGGGTTGTT	150 bp	1.02±0.02
Ocrl	TATGCATGTCACCAGGAGGA	AGCTTCCAGGAAAATGAGCA	150 bp	0.99±0.02
INPP5B	CTGGGGGACCTCAACTACAG	GAGCTCACCCTCTGTGAAGC	156 bp	0.97±0.03
Inpp5b	CTGCCAGGACCATCTTTGAT	TTTCCTTTCCACAGGATTCG	145 bp	0.99±0.04
Lrp2	ATGTCACCTCCATCCTGGTC	GTGGCCACTTGCACATTGTA	148 bp	0.98±0.03
Slc5a2	TTGGGCATCACCATGATTTA	GCTCCCAGGTATTTGTCGAA	164 bp	0.98±0.02
Aqp1	GCTGTCATGTACATCATCGCCCAG	AGGTCATTGCGGCCAAGTGAAT	102 bp	1.02±0.01
Slc34a1	CATCACAGAGCCCTTCACAA	TGGCCTCTACCCTGGACATA	161 bp	0.98±0.03