Autophagosome–lysosome fusion triggers a lysosomal response mediated by TLR9 and controlled by OCRL

Maria Giovanna De Leo1,9, Leopoldo Staiano1,9,10, Mariella Vicinanza1, Alessandro Luciani2, Annamaria Carissimo1, Margherita Mutarelli1, Antonella Di Campoli1, Elena Polishchuk1, Giuseppe Di Tullio1, Valentina Morra1, Elena Levchenko1, Francesca Oltrabella1, Tobias Starborg3, Michele Santoro1, Diego di Bernardo1,1, Olivia Devuyst2, Martin Lowe3, Diego L. Medina1, Andrea Ballabio1,6,7,8 and Maria Antonietta De Matteis1,10

Phosphoinositides (PtdIns) control fundamental cell processes, and inherited defects of PtdIns kinases or phosphatases cause severe human diseases, including Lowe syndrome due to mutations in OCRL, which encodes a PtdIns(4,5)P2 5-phosphatase. Here we unveil a lysosomal response to the arrival of autophagosomal cargo in which OCRL plays a key part. We identify mitochondrial DNA and TLR9 as the cargo and the receptor that trigger and mediate, respectively, this response. This lysosome-cargo response is required to sustain the autophagic flux and involves a local increase in PtdIns(4,5)P2 that is confined in space and time by OCRL. Depleting or inhibiting OCRL leads to an accumulation of lysosomal PtdIns(4,5)P2, an inhibitor of the calcium channel mucolipin-1 that controls autophagosome–lysosome fusion. Hence, autophagosomes accumulate in OCRL-depleted cells and in the kidneys of Lowe syndrome patients. Importantly, boosting the activity of mucolipin-1 with selective agonists restores the autophagic flux in cells from Lowe syndrome patients.

Results

OCRL depletion induces the upregulation of lysosomal genes and morphological alterations of lysosomes

We initially undertook an unbiased approach to uncover how cells respond to the loss of OCRL by analysing the changes in gene

1Telethon Institute of Genetics and Medicine (TIGEM), 80078 Pozzuoli, Naples, Italy. 2Institute of Physiology, University of Zurich, 8057 Zurich, CH, Switzerland. 3Institute of Protein Biochemistry National Research Council, 80131 Naples, Italy. 4Department of Pediatric Nephrology & Growth and Regeneration, University Hospitals Leuven & Katholieke Universiteit, 3000 Leuven, Belgium. 5Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK. 6Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA. 7Jan and Dan Duncan Neurological Research Institute, Texas Children’s Hospital, Houston, Texas 77030, USA. 8Medical Genetics, Department of Translational Medicine, Federico II University, 80131 Naples, Italy. 9These authors contributed equally to this work.

Received 8 August 2015; accepted 9 June 2016; published online 11 July 2016; corrected online 18 July 2016; DOI: 10.1038/ncb3386
Figure 1  OCRL depletion/mutation induces upregulation of lysosomal genes and morphological changes in lysosomes. (a) Volcano plot of OCRL-KD gene expression data. Horizontal black line: –log\(_{10}\) of false discovery rate (FDR; significance threshold 0.05); vertical black lines: log\(_{2}\) fold change (1.3-fold threshold). Nine hundred and ten genes upregulated above (red) and 630 genes downregulated below (green) the threshold are shown. The black dots indicate 24 upregulated (35 probe sets) out of the 194 lysosomal genes annotated in the Lysoplex list\(^\text{30}\). (b) Gene set enrichment analysis of the data in a. Subcellular compartments, except lysosomes, were defined according to the Human Protein Atlas. Lysosomal genes were defined according to Lysoplex\(^\text{30}\) and excluded from the other categories. P values were calculated as described in the Methods. (c–e) Lysosomal enlargement in OCRL-depleted HK-2 cells, in PTCs of Lowe syndrome patients, and in the proximal pronephric tubules of \(ocrl^{-/-}\) zebrafish embryos. (c) Representative images of control and OCRL-KD cells immunostained with an anti-LAMP1 antibody (top panels, scale bars, 10\(\mu\)m) or immunoelectron microscopy with an anti-LAMP1 antibody (bottom panels, scale bars, 250 nm). (d) PTCs obtained from the urine of healthy control subjects (CTRL) and Lowe syndrome patients were processed as in a. The black arrows indicate LAMP1-positive structures. (e) Confocal transverse sections of zebrafish proximal pronephric tubules from 72 hpf GFP–LAMP1-expressing WT and \(ocrl^{-/-}\) mutant embryos\(^\text{5}\) labelled with an anti-GFP antibody. The white dashed lines indicate the outline of pronephric tubules (top panels). Scale bars, 5\(\mu\)m. Block face scanning electron microscopy images of transverse sections through proximal pronephric tubules from 72 hpf WT or \(ocrl^{-/-}\) embryos (bottom panels). Scale bars, 5\(\mu\)m. Graphs show the morphometric analysis of LAMP1-positive structures in c \((n=81 \text{ (CTRL) and } n=126 \text{ (OCRL-KD) structures pooled from three independent experiments})\) and d \((n=102 \text{ (CTRL) and } n=84 \text{ (Lowe) structures pooled from three independent experiments})\). In e, the external circumference of each lysosomal compartment (electron-dense oval or spherical membrane-enclosed organelle) was manually traced in each section and the average calculated. \(n=30 \text{ sections (WT); } n=30 \text{ sections (} ocrl^{-/-}\text{)}\) (10 sections analysed for three kidneys per category). Data are presented as means ± standard deviation (s.d.) \(^{\text{**}* P < 0.001,} \) calculated by two-tailed Student’s t-test.

expression caused by the depletion of OCRL in human PTCs, the cells that are primarily compromised in Lowe syndrome (Supplementary Fig. 1a–c and Supplementary Table 1).

We found that one of the gene classes most significantly upregulated after OCRL depletion coded for lysosomal proteins (Fig. 1a,b and Supplementary Fig. 2a). This upregulation was
accompanies by the nuclear translocation of the transcription factor TFEB (Supplementary Fig. 2b–d), a master regulator of lysosomal biogenesis that is activated under conditions that require increased degradation efficiency or induce lysosomal dysfunction. The activation of TFEB and the presence of morphologically abnormal lysosomes in OCRL-depleted cells, in PTCs from Lowe syndrome patients, and in a recently described zebrafish model of Lowe syndrome\(^5,10\) (Fig. 1c–e) led us to investigate the role of OCRL at lysosomes.

**OCRL is recruited to lysosomes in response to autophagosome–lysosome fusion in an AP2- and clathrin-dependent manner**

As mentioned above, OCRL has been reported to associate with clathrin-coated pits, clathrin-coated vesicles, early endosomes, the TGN, and the primary cilium\(^1\). However, this distribution pattern apparently contrasts with the first reported localization of OCRL, which indicated that OCRL associates with lysosomes that were however loaded with sucrose\(^11\). We reasoned that this apparent discrepancy might be due to the different loading state of lysosomes and therefore analysed the distribution of OCRL under a condition that induces a lysosomal cargo load such as starvation, during which lysosomes receive autophagosomal cargoes. We confirmed that OCRL associates mainly with early endosomes and the TGN (Supplementary Fig. 1b) and only occasionally co-localizes with the lysosomal marker LAMP1 under steady-state conditions (Fig. 2a,b).

However, it massively translocates to lysosomes on induction of autophagy (Fig. 2a,b). The observation that this translocation was diminished when the fusion of autophagosomes with lysosomes was inhibited by depleting key components of the tethering (VPS16) and fusion (STX17) machinery or interfering with microtubule dynamics (vinblastine treatment)\(^12-14\) (Fig. 2a,b) indicated that the fusion of autophagosomes with lysosomes triggers the recruitment of OCRL to lysosomes and prompted us to look for other components and for the signalling pathways mediating this response.

We first explored the involvement of clathrin and AP2, two main recruiters of OCRL to cellular membranes\(^15,16\), and found that mutations in the AP2- and/or clathrin-binding sites of OCRL impaired the translocation of OCRL to lysosomes\(^16\) (Fig. 2c). Confirming the importance of these two binding sites, INPP5B, a PtdIns(4,5)P\(_2\) 5-phosphatase that is highly similar to OCRL but lacks the AP2-and clathrin-interacting motifs\(^16\), is not recruited to lysosomes in response to the delivery of autophagic cargo (Fig. 2c).

**Autophagosome–lysosome fusion triggers an increase in lysosome PtdIns(4,5)P\(_2\) and the recruitment of AP2 and clathrin to lysosomes**

Prompted by the observation that AP2 and clathrin were required for the association of OCRL with lysosomes in response to autophagosome–lysosome fusion, we followed their localization during the course of the autophagy process. AP2 and clathrin have been reported to associate with lysosomes\(^17\) and to be required for lysosome reformation at the end of autophagy on mTOR signalling reactivation\(^17,18\). We found that AP2 and clathrin are in fact recruited to lysosomes early during the autophagy process at a stage when mTOR is still inactive, and in a manner that is concomitant with and dependent on the fusion of autophagosomes with lysosomes and a local increase in PtdIns(4,5)P\(_2\), produced by PI(4)KIII\(\beta\), a kinase that preserves lysosomal identity\(^19\), and the kinases PIP5K1\(\alpha\) and \(\beta\) (Fig. 3a–c and Supplementary Fig. 3a, b).

To search for signalling pathways mediating the lysosomal response to autophagosome fusion, we first considered the lysosome-based mTOR signalling\(^20\). However, multiple lines of evidence indicated that the local increase in lysosomal PtdIns(4,5)P\(_2\) and the recruitment of OCRL are not dependent on mTOR activation or inactivation: first, OCRL is more associated with lysosomes (as compared with steady state) under conditions in which mTOR is either silent or fully active (Fig. 3b and Supplementary Fig. 3c); second, the overall time course of OCRL translocation to lysosomes does not parallel that of mTOR inactivation–reactivation during nutrient deprivation-induced autophagy followed by nutrient re-addition (Fig. 3b); third, it is not starvation (and thus mTOR inactivation) per se but the arrival of autophagosomal cargo to lysosomes that induces this response (Fig. 3c); fourth, OCRL translocation can be seen using agents that induce autophagy and inactivate mTOR (torin 1) or induce autophagy without affecting mTOR activity (Tat–Beclin 1)\(^21\) (Supplementary Fig. 3d, e).

**TLR9 mediates the lysosomal response to autophagosome fusion**

We therefore considered the involvement of other endolysosome-based signalling pathways, such as members of the family of toll-like receptors (TLRs) that are present in immune as well as in non-immune cells (including kidney PTCs\(^23-25\)) and recognize both exogenous and endogenous macromolecules\(^26-28\). We depleted TLR family members that can signal from endolysosomes (that is, TLR3, TLR4, TLR7, TLR9) and found that the response to autophagosome–lysosome fusion was blunted—in terms of PtdIns(4,5)P\(_2\) generation, and AP2, clathrin and OCRL recruitment—by selectively depleting or pharmacologically inhibiting TLR9 (Fig. 4a–c). TLR9 resides mainly in the endoplasmic reticulum (ER) at steady state (with only a small fraction of receptors present in the endolysosomal compartment) but on arrival of TLR9 ligands into lysosomes it translocates to lysosomes via mechanisms that are only partially understood and that involve COPII-dependent ER export, the chaperone UNC93B1, and the small GTPase ARF3\(^29,30\). In line with the notion that TLR9 activation is accompanied by its translocation to lysosomes, we found that TLR9 translocates from the ER to lysosomes on autophagy induction (Fig. 4d and Supplementary Fig. 4a) in a fashion that is dependent on autophagosome–lysosome fusion and on the presence of the TLR9 chaperone UNC93B1 (Fig. 4d).

**TLR9 is activated by mitochondrial DNA released into lysosomes by autophagosomes**

The ligands for TLR9 are unmethylated CpG motifs that are found in bacterial DNA and in mitochondrial DNA (mtDNA)\(^31\). Indeed, the activation of TLR9 in response to mitochondrial damage and increased autophagy has been reported recently in non-immune cells such as cardiomyocytes\(^32\) and hepatocytes\(^33\). We thus hypothesized that the autophagic cargo that triggers the TLR9-mediated lysosomal response might be mtDNA released into lysosomes by autophagosomes. In support of our hypothesis, depleting
Figure 2 Autophagosome–lysosome fusion induces an AP2- and clathrin-dependent recruitment of OCRL to lysosomes. (a) Fusion of autophagosomes with lysosomes recruits OCRL to lysosomes. HK-2 cells were incubated in growth medium, or in HBSS for 3 h (–), or in HBSS for 3 h after impairment of autophagosome–lysosome fusion by siRNA-mediated knockdown of the HOPS component VPS16 (ref. 12) or of the autophagosomal SNARE STX17 (ref. 14). Cells were stained for OCRL and LAMP1 (a lysosomal marker). The bottom panels are enlargements of the outlined areas and fluorescence intensity profiles in the green and red channels of the regions underneath the white lines. Scale bars, 10 μm. (b) Quantification of OCRL association with lysosomes under the conditions described in (a), or after the addition of vinblastine (20 μM) that also inhibits autophagosome–lysosome fusion13. The percentage of LC3 structures that are positive for OCRL, the percentage of LC3 structures that are positive for LAMP1 (as a measure of the arrival of autophagosomal cargo to lysosomes), and the average number of LC3 structures per cell are reported. Values are means ± s.d. of n=450 cells pooled from three independent experiments. NS, not significant. (c) Representative images and quantification of the co-localization of OCRL-WT, the AP2 (OCRL-F151S), clathrin (OCRL-I74N) or AP2–clathrin triple (OCRL-X3) binding mutants, and INPP5B with LAMP1. Insets, enlargements of the outlined areas; bottom panels, fluorescence intensity profiles in the green and red channels of the regions underneath the white lines. Scale bars, 10 μm.

Data represent the percentage of total LAMP1 structures positive for each OCRL form or for INPP5B. Means ± s.d. n=200 cells pooled from three independent experiments; n=100 OCRL-X3-transfected cells pooled from three independent experiments. P values calculated by one-way analysis of variance (ANOVA) with Tukey’s post hoc test.

mitochondrial DNA (by two independent means, Supplementary Fig. 4b, c) or depleting DNaseII (the lysosomal DNase required for TLR9 activation34) inhibited the lysosomal response (Fig. 4e and Supplementary Fig. 4d), while inducing mitophagy with FCCP (a mitochondrial uncoupler that induces mitochondrial damage35-37) or activating TLR9 with synthetic agonists mimicking unmethylated
CpG motifs elicited the lysosomal response in terms of an increase in lysosomal PtdIns(4,5)P₂ and the recruitment of AP2, clathrin and OCRL (Fig. 4f).

We investigated the signalling cascade triggered by the activation of TLR9 in response to mtDNA and found that known components of the TLR9 signalling and trafficking pathway—such as TIRAP, which also translocated to lysosomes (Supplementary Fig. 4e), MyD88, IRAK4 and UNC93B1—were involved (Supplementary Fig. 4f). We next asked how the activation of TLR9 could lead to the local increase in PtdIns(4,5)P₂. Although a link between the activation of TLR and PtdIns(4,5)P₂ generation has been previously reported, the underlying mechanisms have remained unexplored so far. We found that the activation of TLR9 is accompanied by and required for the translocation of PIP5Kα and β to lysosomes (Fig. 4g, h) and that TLR9 belongs to a molecular complex containing also PIP5Kα, since the two proteins can be co-immunoprecipitated (Fig. 4i).

The TLR9-mediated lysosomal cargo response sustains the autophagic flux

Altogether, the above data unmasked a regulatory circuit in lysosomes that is activated by autophagic cargo and prompted us to ask what might be the purpose(s) of this circuit and what is the specific role of OCRL in it. We found that this circuit controls local (that is, lysosomal) and transcriptional responses. Locally, it is required for lysosomal homeostasis since lysosomes enlarge and accumulate internal membranes on depletion or inhibition...
Figure 4 TLR9 stimulated by mtDNA released into lysosomes by autophagosomes mediates the lysosome-cargo response. (a) Co-localization of LAMP1 and OCRL in control cells, cells knocked down for the indicated TLRs or treated with the TLR9 antagonist ODN TTAGGG (0.5μM). Mean values ± s.d. n=100 cells per condition pooled from three independent experiments. (b) OCRL association with lysosomes in CTRL, TLR9-KD and ODN TTAGGG-treated starved cells. Insets, enlargements of the outlined areas. (c) Effects of TLR9 depletion/inhibition on the lysosomal cargo response. Mean values ± s.d. n=450 cells per condition pooled from three independent experiments. (d) Co-localization of TLR9–YFP with LAMP1 under the indicated conditions. Insets, enlargements of the outlined areas. Mean values ± s.d. n=100 cells per condition pooled from three independent experiments. P<0.01 for starved versus fed CTRL cells and starved CTRL cells versus starved VPS16-KD and UNC93B1-KD cells. (e) Co-localization of PtdIns(4,5)P2 and the indicated proteins with LAMP1 in starved CTRL cells (–), or after DNAseII-KD, or after mtDNA depletion (RhoZero) obtained as specified in Supplementary Fig. 4b. Mean values ± s.d. n=200 cells per condition pooled from three independent experiments. (f) HK-2 cells untreated or treated with FCCP (10μM) alone or with TLR9 antagonist (ODN TTAGGG), or treated with TLR9 agonists (ODN 2216 and ODN 2395) and stained for PtdIns(4,5)P2 and the indicated proteins. Mean values ± s.d. n=100 cells per condition pooled from three independent experiments. (g) CTRL and TLR9-KD starved cells stained for LAMP1 and for PIP5K1α or PIP5K1β. Insets, fluorescence intensity profiles in the green and red channels of the regions underneath the white lines. (h) Quantification of PIP5K1α or PIP5K1β co-localization with LAMP1. Mean values ± s.d. n=100 cells per condition pooled from three independent experiments. (i) Cell lysates from non-transfected or TLR9–YFP-transfected cells were immunoprecipitated with anti-GFP antibody and analysed by western blot. Results representative of five independent experiments. An unprocessed original scan of the blot is shown in Supplementary Fig. 9. P values are indicated, calculated by one-way ANOVA with Tukey’s post hoc test. Scale bars, 10μM. Statistical source data can be found in Supplementary Table 2.
Figure 5 TLR9 is required for lysosomal homeostasis and for efficient autophagic flux. (a) Control, TLR9-KD and TLR9 antagonist-treated cells were stained for LAMP1. (b) Representative image of autophagosomes (black asterisk) and lysosomes (labelled with anti-LAMP1 antibodies, black arrows) in TLR9-KD cells. The graph shows the size distribution of LAMP1-positive structures; n = 85 (CTRL) and n = 80 (TLR9-KD) structures pooled from three independent experiments. The table reports the morphometric analysis: autophagosomes (AV); autolysosomes (AL); multivesicular bodies and electron-dense lysosomes (MVB + Ly); multilamellar bodies (MLB). P ≤ 0.01 for AV and AL in TLR9-KD relative to CTRL cells. Mean values ± standard error of the mean (s.e.m.). (c) Cell lysates were probed with the indicated antibodies. BafA1, bafilomycin A1; β-Actin was used as a loading control. Graphs show p62/actin and LC3-II/actin ratios. Mean values ± s.d. n = 3 lysates per condition pooled from three independent experiments. Unprocessed original scans of the blots are shown in Supplementary Fig. 9. (d) Control (CTRL), TLR9-KD, and TLR9-antagonist-treated cells were fixed and immunostained with an anti-LC3 antibody. The number of LC3-positive structures per cell is reported in the graph. Mean values ± s.d. n = 450 cells per condition pooled from three independent experiments. (e) Cells expressing mRFP–GFP tandem-tagged LC3 were incubated in growth medium, in HBSS for 3 h with or without a protease inhibitor cocktail (PI) or the TLR9 antagonist ODN TTAGGG (0.5 μM) as indicated. Insets are enlargements of the outlined areas. The table reports the means (±s.d.) of AV and AL. n = 60 cells per condition pooled from three independent experiments. P < 0.001 for TLR9-KD and ODN TTAGGG-treated cells versus CTRL cells. (f) NF-κB nuclear localization. Mean values ± s.d. n = 400 cells per condition pooled from three independent experiments. (g) Quantitative PCR analysis of IL-6 and IFN-β mRNA levels in the indicated conditions. Mean values ± s.d. n = 3 RNAs per condition pooled from three independent experiments. P values are indicated, and were calculated by one-way ANOVA with Tukey’s post hoc test except in b where Student’s t-test was used. Statistical source data can be found in Supplementary Table 2. Scale bars, 10 μm in a,d,e and 500 nm in b.
defective recycling of the autophagic SNARE syntaxin1714 from autolysosomes (Supplementary Fig. 4g). Concomitantly with this local lysosomal response, TLR9 stimulation by mtDNA triggers the canonical TLR9-dependent signalling cascade involving the activation and nuclear translocation of the transcription factor NF-κB and an increase in the transcription of proinflammatory cytokine and interferon beta genes (Fig. 5f,g). This indicates that the mtDNA released into lysosomes during autophagy has the potential to trigger an inflammatory response. We envisage that this response is usually self-limiting due to the complete degradation of oligonucleotides containing unmethylated CpG motifs by lysosomal exonucleases, nucleotidases and phosphatases but might become sustained under conditions of prolonged stress and/or impaired degradative capacity, such as during lysosomal storage disorders, which, in fact, are often accompanied by chronic inflammation40.

**OCRL controls the autophagy flux through its 5-phosphatase activity**

We then explored the role of OCRL in the lysosomal cargo response and its relevance for the manifestations of Lowe syndrome. We found that OCRL-depleted cells have a higher number of mature

Figure 6 Autophagosomes accumulate in OCRL-depleted cells and PTCs and kidneys from Lowe syndrome patients. (a) LC3 in cells with or without the OCRL inhibitor YU142670 (ref. 43) and OCRL-KD cells with or without siRNA-resistant wild-type or catalytically inactive (V527D) OCRL. Insets, transfected cells. (b) Quantification of LC3 structures in the cells described in a. Red line, mean ± s.d. n = 200 cells per condition pooled from four independent experiments. NS, not significant. (c) Ultrastructure and quantification of AV (black arrow), AL (white arrows), MVB+Ly (white arrowheads) and MLB in control and OCRL-KD cells labelled for LAMP1. P < 0.05 for AV and MLB in OCRL-KD versus CTRL cells. Mean ± s.e.m. n = 14 (CTRL), n = 18 (OCRL-KD) fields pooled from three independent experiments. (d) LC3 and LAMP1 in CTRL, OCRL-KD and YU142670-treated cells. Bottom panels, enlargements of the outlined areas. Means ± s.d. of LC3 and LAMP1 co-localization (mean ± s.d.) n = 120 cells per condition pooled from three independent experiments. P < 0.001 for LC3 structures in OCRL-KD and YU142670-treated versus fed CTRL and for LC3–LAMP1 co-localization in starved CTRL versus starved OCRL-KD. (e) mRFP–GFP–LC3 in control, OCRL-KD and YU142670-treated cells. Arrows, AL. Quantification of AV and AL. Mean ± s.d. n = 150 cells per condition pooled from three independent experiments. P < 0.001 for AV and AL in OCRL-KD and YU142670-treated cells versus CTRL cells. (f) Autophagosomes accumulate in aquaporin (AQP1)-positive proximal tubules. 4,6-Diamidino-2-phenylindole (DAPI) (blue), nuclei. Means ± s.e.m., n = 50 proximal tubules pooled from ten fields per biopsy from three Lowe syndrome patients and four control subjects. *P < 0.025. (g) LC3 in PTCs from healthy subjects or Lowe syndrome patients transfected or not with OCRL-WT or OCRL-V527D. Insets, transfected cells outlined in the main image. Mean ± s.d. of LC3-positive structures per cell; n = 150 cells per condition pooled from three independent experiments. P < 0.001 for Lowe versus CTRL and for Lowe+OCRL-WT versus Lowe. P < 0.01 for Lowe+OCRL-V527D versus Lowe. P values in b,d,g were calculated by one-way ANOVA with Tukey’s post hoc test, and by Student’s t-test in c. Statistical source data can be found in Supplementary Table 2. Scale bars, 10 μm in a,d,e,g; 250 nm in c; 50 μm in f.
Autophagy flux is impaired due to PtdIns(4,5)P₂-mediated inhibition of MCOLN1 activity in OCRL-depleted cells. (a) Mock (CTRL), YU142670-treated and OCRL-KD (Sigma (S) or Ambion (A) siRNA pools) cells were incubated in HBSS for 3 h and labelled with anti-LAMP1 (green) and anti-LAMP1–PtdIns(4,5)P₂ (red) antibodies. White arrows indicate structures positive for both markers. (b) Quantification of PtdIns(4,5)P₂-LAMP1 co-localizing structures in control, YU142670-treated, OCRL-KD (–), OCRL-KD plus PIP5K1α-KD, and OCRL-KD plus PIP5K1α-KD cells. Mean values ± s.d. n = 200 cells per condition pooled from four independent experiments. (c) Quantification of LC3-positive structures in the cells described in b. Red line, mean values. (d) OCRL depletion impairs MCOLN1-dependent calcium release. Control or OCRL-KD cells loaded with the Ca²⁺-sensitive dye FuraRed were treated with 50 or 200 μM of the MCOLN1 agonist SF-51 (ref. 47). The ratio of fluorescence excited at 458 and 488 nm was measured and normalized against F₀ (time point before drug addition, black arrow). Plots correspond to the average of the response of n = 20 cells from a single experiment, which was independently repeated three times. (e) Representative images and quantification of MCOLN1 and OCRL co-localization in cells transfected with wild-type OCRL or the AP2-defective binding mutant (OCRL-F151S) along with MCOLN1–Myc. Right, co-localization of OCRL with MCOLN1. Mean values ± s.d. n = 100 cells per condition pooled from three independent experiments. (f) MCOLN1-KD cells transfected with OCRL-WT–GFP were treated with HBSS for 3 h and stained for LAMP1 (red). Right, quantification of OCRL co-localization with LAMP1. Mean values ± s.d. n = 100 cells per condition pooled from three independent experiments. (g) Cell lysates from HK-2 cells were immunoprecipitated using an anti-MCOLN1 antibody and analysed by western blot (WB) with anti-MCOLN1 or anti-OCRL antibodies. The results are representative of five independent experiments. An unprocessed original scan of the blot is shown in Supplementary Fig. 9. Parallel samples were analysed by LC-MS/MS and peptides significantly matching human OCRL (Q01968-2) were found in anti-MCOLN1 immunoprecipitates but not in control-IgG immunoprecipitates. P values were calculated by one-way ANOVA with Tukey’s post hoc test. Statistical source data can be found in Supplementary Table 2. Scale bars, 10 μm.
induced an accumulation of autphagosomes (Fig. 6a,b) while the autophagic flux could be restored by expressing wild-type (WT) but not catalytically inactive OCRL in OCRL-defective PTCs or in PTCs from Lowe syndrome patients (Fig. 6a,b,g). Of note, we detected an excess of lysosomal PtdIns(4,5)P_2 in OCRL-depleted cells (Fig. 7a,b and Supplementary Fig. 5e, f) that could be rescued by re-expressing WT, but not catalytically inactive, OCRL (Supplementary Fig. 5f) or by lowering PtdIns(4,5)P_2 production through the depletion of PIP5K α or β (Fig. 7b). Notably, these two independent PtdIns(4,5)P_2-reducing approaches were both effective in rescuing the autophagy flux in OCRL-depleted cells (Figs 6a,b and 7c), indicating that the excess lysosomal PtdIns(4,5)P_2 had a causative role in impairing autophagosome–lysosome fusion in OCRL-depleted cells.

**OCRL is required to preserve the activity of MCOLN1**

We searched for the target(s) of the inhibitory effect of PtdIns(4,5)P_2 on autophagosome–lysosome fusion. One interesting candidate was mucolipin-1 (MCOLN1, also referred as TRPML1), a lysosomal calcium channel required for lysosome fusion with autophagosomes whose activity is stimulated by PtdIns(3,5)P_2 but inhibited by PtdIns(4,5)P_2 (refs 44–46). Indeed, we found that MCOLN1-dependent calcium release was compromised—that is, no response at low concentrations and slower response at high concentrations of MCOLN1 agonist—in OCRL-KD cells7 as compared with control cells (Fig. 7d). We also obtained independent lines of evidence indicating that MCOLN1 and OCRL interact: firstly, the overexpression of MCOLN1 recruits WT but not the AP2-defective binding mutant of OCRL to lysosomes (Fig. 7e); secondly, MCOLN1 is required for OCRL translocation to lysosomes (Fig. 7f); thirdly, the two proteins can be co-immunoprecipitated (Fig. 7g). Thus, our results indicate that both MCOLN1 (Fig. 7f) and AP2 (Fig. 2c) are required for the lysosomal recruitment of OCRL, but that neither of them on its own is sufficient since AP2-defective OCRL mutants are no longer recruited to lysosomes even in cells overexpressing MCOLN1 (Fig. 7e),
and AP2-competent OCRL cannot be recruited to lysosomes in the absence of MCOLN1 (Fig. 7f). This combined requirement indicates that a mechanism of coincidence detection drives and calibrates in time and space the recruitment of OCRL to lysosomes ensuring that OCRL is recruited to lysosomal regions/domains containing both MCOLN1 and AP2.

Boosting MCOLN1 activity rescues the autophagic flux in Lowe syndrome patient PTCs

Since OCRL depletion did not have an impact on MCOLN1 localization (Supplementary Fig. 7a), we concluded that the MCOLN1 dysfunction in OCRL-depleted cells was due to reduced activity caused by an excess of PtdIns(4,5)P₂. Hence, we reasoned that boosting the activity of MCOLN1 in OCRL-depleted cells might rescue lysosomal function. To this end we used two independent approaches: the overexpression of MCOLN1 (the WT form and MCOLN1-3A, a mutant MCOLN1 unable to bind PtdIns(4,5)P₂; ref. 44) or the use of a MCOLN1 activator at a concentration that is effective in inducing lysosomal calcium release in OCRL-depleted cells (Fig. 7d). We observed that both the overexpression of MCOLN1, more potently in its PtdIns(4,5)P₂-insensitive form (MCOLN1-3A), and treatment with the MCOLN1 activator SF-51 (which we further characterized for its specificity, Supplementary Fig. 7b) rescued the defects in autophagic flux in OCRL-depleted cells (Fig. 8a,b). Importantly, the MCOLN1 activator also rescued the impaired autophagic flux in PTCs from Lowe syndrome patients (Fig. 8c,d), thus indicating MCOLN1 as a possible drug target for the treatment of Lowe syndrome.

DISCUSSION

Here we have unveiled a heretofore unknown ability of lysosomes to sense the arrival of autophagic cargo and at the same time a pathophysiological pathway in Lowe syndrome (Supplementary Fig. 8). The mtDNA released by autophagosomes into lysosomes stimulates TLR9, which activates its known downstream effectors, such as the transcription factor NF-κB (Supplementary Fig. 8), but also induces a local increase in PtdIns(4,5)P₂. The observation that OCRL, a 5-phosphatase, is recruited almost simultaneously with PIP5Ks demonstrates the need for a strict spatial and temporal restriction of PtdIns(4,5)P₂ levels in lysosomes. The local increase in PtdIns(4,5)P₂ is needed for the recruitment of AP2 and clathrin. We envisage that AP2-mediated clathrin recruitment controls distinct budding events in lysosomes in distinct stages of autophagy. During ongoing autophagy (this report) it may mediate the recycling of components, such as syntaxin 17, needed to sustain an efficient autophagic flux, while at the end of autophagy, as shown in ref. 19, it mediates autophagic lysosome reformation to regenerate primary lysosomes that have been consumed through their fusion with autophagosomes. PtdIns(4,5)P₂, which is required for these budding events, has to be nevertheless restricted to limited domains in the lysosome since it can act as an endogenous inhibitor of the calcium channel MCOLN144,48, This is the task of OCRL, which interacts with MCOLN1 and which ensures PtdIns(4,5)P₂-free microdomains around MCOLN1 (Supplementary Fig. 8). Indeed, the uncontrolled increase in lysosomal PtdIns(4,5)P₂ that results from the loss of function of OCRL inhibits MCOLN1 activity and, consequently, the autophagic flux, inducing the accumulation of autophagosomes that is observed in cells and, notably, in the kidney of Lowe syndrome patients. The lysosome dysfunction and the impaired autophagic flux are bound to play a pivotal role in the progression of renal damage, a so far poorly understood process, and, possibly, also in the generation of central nervous system-related symptoms and of neuropathological signs in Lowe syndrome patients.6,7,49 Finally, and most importantly, MCOLN1 emerges as an interesting drug target since MCOLN1 agonists are able to restore the autophagosomal flux in cells from Lowe syndrome patients.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of this paper.

ACKNOWLEDGEMENTS

We thank S. Aydin (UCL Brussels), N. Demoulin (UCL Brussels), M.-C. Gabler (INSERM Paris), G. Montini (Univ. Bologna), U. Querfeld (Charité Berlin), B. Rudolph (Charité Berlin) and F. Schaeffer (Univ. Heidelberg) for providing renal biopsies from patients with Lowe syndrome and appropriate controls, C. Wilson, F. Emma, C. Settembre, N. R. Pierri and R. Venditti for insightful discussions, G. Diez-Roux for critical reading of the manuscript, the TIGEM Bioinformatics Core and M. Failli for the analysis of the gene expression profiles, the Advanced Microscopy and Imaging Core of TIGEM for the EM analysis, and the Central Proteomics Facility, Sir William Dunn Pathology School, Oxford University. M.A.D.M. acknowledges the support of Telethon (grant TGM11CB1), the Italian Association for Cancer Research (AIRC, grant IG2013_14761), European Research Council Advanced Investigator grant no. 670881 (SYSMET), Associazione Italiana Sindrome di Lowe (AISLO), Lowe Syndrome Association, USA (LSA) and Programma Operativo Nazionale (PON) 01_08862. M.G.D.L. received a Fellowship from AIFRC. E. Levchenko was supported by the fund for Scientific Research, Flanders (EWO. Vlaanderen) grant 180110N. F.O. was supported by a grant from the UK Lowe Syndrome Trust (NoMUML1010) awarded to M.L. T.S. was supported by the Wellcome Trust (088785/Z/09/Z). O.D. was supported by the Fonds National de la Recherche Scientifique and the Fonds de la Recherche Scientifique Médicale (Brussels, Belgium); the European Community’s Seventh Framework Programme under grant agreement no. 305608 (EURenOmicS); the Cystinosis Research Foundation (Irvine, California, USA); the Rare Disease Initiative Zürich (RADIZ), and the Swiss National Science Foundation project grant 310030_146490 (O.D. and A.L.).

AUTHOR CONTRIBUTIONS

M.A.D.M. supervised the entire project; M.A.D.M. and L.S. wrote the manuscript with comments from all co-authors; M.G.D.L. and L.S. designed and conducted the experiments of immunolocalization, induction of autophagy, western blots, and immunoprecipitation with the help of V.M., M.S. and G.D.T.; M.V. and A.D.C. performed the initial experiments on PTCs from Lowe syndrome patients; M.S. and G.D.T. designed the strategy and produced plasmid vectors for the different constructs used in the study; M.S. and G.D.T. prepared recombinant proteins and anti-OCRL antibodies; E.P. designed and conducted the experiments for electron microscopy; D.L.M. performed the calcium measurements; F.O., T.S. and M.L. performed the study in the ocr−/− zebrafish model; E.L. isolated the PTCs from the urine of Lowe syndrome patients; A.L. performed the study of immunostaining in kidney biopsies under the supervision of O.D.; A.C., M.M. and D.D.B. performed the analysis of the gene expression profiles; A.B. provided the knowledge and material needed for the study of autophagy and TFE8.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3386
Reprints and permissions information is available online at www.nature.com/reprints

1. Attree, O. et al. The Lowe’s oculocerebrorenal syndrome gene encodes a protein highly homologous to inositol polyphosphate-5-phosphatase. Nature 358, 239–242 (1992).
2. Staiano, L., De Leo, M. G., Persico, M. & De Matteis, M. A. Mendelian disorders of PI metabolizing enzymes. Biochim. Biophys. Acta 1851, 867–881 (2015).
3. Mehta, Z. B., Pietka, G. & Lowe, M. The cellular and physiological functions of the Lowe syndrome protein OCRL1. Traffic 15, 471–487 (2014).
et al. 2016 Macmillan Publishers Limited. All rights reserved

ARTICLES

1. Nandez, R. et al. A role of OCRL in clathrin-coated pit dynamics and uncoating revealed by studies of Lowe syndrome cells. *Elife* 3, e02975 (2014).

2. Vicinanza, M. et al. OCRL controls trafficking through early endosomes via Phlins,Lip2-dependent regulation of endosomal actin. *EMBO J.* 30, 4970–4985 (2011).

3. Carvalho-Neto, A., Ono, S. E., Cardoso Gde, M., Santos, M. L. & Celdonio, I. Oculocerebrorenal syndrome of Lowe: magnetic resonance imaging findings in the first six years of life. *Arq. Neuropsiquiatr.* 67, 305–307 (2009).

4. Allmendinger, A. M., Desai, N. S., Burke, A. T., Viswanadhan, N. & Prabhu, S. Neuroimaging and renal ultrasound manifestations of Oculocerebrorenal syndrome of Lowe. *J. Radiol. Case Rep.* 8, 1–7 (2014).

5. Settembre, C. et al. TFEB links autophagy to lysosomal biogenesis. *Science* 332, 1429–1433 (2011).

6. Ottrelabia, F. et al. The Lowe syndrome protein OCRL1 is required for endocytosis in the zebrafish pronephric tubule. *PloS Genet.* 11, e1005058 (2015).

7. Ramirez, I. B. et al. Impaired neural development in a zebrafish model for Lowe syndrome. *Hum. Mol. Genet.* 21, 1744–1759 (2012).

8. Jiang, P. et al. Impaired neural development in a zebrafish model for Lowe syndrome. *Hum. Mol. Genet.* 21, 1744–1759 (2012).

9. Zhang, X., Hartz, P. A., Philip, E., Racusen, L. C. & Majerus, P. W. Cell development of tubulointerstitial injury in systemic lupus. *Nat. Cell Biol.* 13, 2621–2628 (2011).

10. Ramirez, I. B. et al. Impaired neural development in a zebrafish model for Lowe syndrome. *Hum. Mol. Genet.* 21, 1744–1759 (2012).

11. Zhang, X., Hartz, P. A., Philip, E., Racusen, L. C. & Majerus, P. W. Cell development of tubulointerstitial injury in systemic lupus. *Nat. Cell Biol.* 13, 2621–2628 (2011).

12. Jiang, P. et al. The HOPS complex mediates autophagosome–lysosome fusion through interaction with syntaxin 17. *Mol. Biol. Cell* 25, 1327–1337 (2014).

13. Kochl, R., Hu, X. W., Chan, E. Y. & Tooez, S. A. Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. *Traffic* 7, 129–145 (2006).

14. Itakura, E. & Mizushima, N. Clathrin and phosphatidylinositol-4,5-bisphosphate regulate mitochondrial priming. *J. Cell Biol.* 208, 2305–2314 (2013).

15. Yen, Y. et al. A PH domain within OCRL bridges clathrin-mediated membrane trafficking to phosphoinositide metabolism. *EMBO J.* 28, 1831–1842 (2009).

16. Trab, L. M. et al. AP-2-containing clathrin coats assemble on mature lysosomes. *J. Cell Biol.* 135, 1801–1814 (1996).

17. Yu, L. et al. Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature* 465, 942–946 (2010).

18. Rong, Y. et al. Clathrin and phosphatidylinositol-4,5-bisphosphate regulate autophagic lysosome reformation. *Nat. Cell Biol.* 14, 924–934 (2012).

19. Sriram, S. et al. The lipid kinase PI4KIIIβ preserves lysosomal identity. *EMBO J.* 32, 324–339 (2013).

20. Zoncu, R. et al. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H+-ATPase. *Science* 334, 678–683 (2011).

21. Shoji-Kawata, S. et al. Identification of a candidate therapeutic autophagy-inducing peptide. *Nature* 494, 201–206 (2013).

22. Mudalair, H. et al. The role of Toll-like receptor proteins (TLR) 2 and 4 in mitochondria and heart failure. *Nature* 485, 251–255 (2012).

23. Curchin, E. H. et al. Experimental sepsis-induced mitochondrial biogenesis is dependent on autophagy, TLR4, and TLR9 signaling in liver. *FASEB J.* 27, 4703–4711 (2013).

24. Dawar, S. et al. Cutting edge: DNase II deficiency prevents activation of autoreactive B cells by double-stranded DNA endogenous ligands. *J. Immunol.* 194, 1403–1407 (2010).

25. Chen, G. et al. A regulatory signaling loop comprising the PGAM5 phosphatase and CK2 controls receptor-mediated autophagy. *Mol. Cell* 54, 362–377 (2014).

26. Ding, W. X. et al. Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J. Biol. Chem.* 285, 27879–27890 (2010).

27. Narendra, D., Tanaka, A., Suen, D. F. & Youle, R. J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *Cell Biol.* 183, 795–803 (2006).

28. Nguyen, T. T. et al. Phosphatidylinositol 4-phosphate 5-kinase α facilitates Toll-like receptor 4-mediated microglial inflammation through regulation of the Toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP) location. *J. Biol. Chem.* 288, 5645–5659 (2013).

29. Kimura, S., Noda, T. & Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 3, 452–460 (2007).

30. Platt, N. et al. Immune dysfunction in Niemann-Pick disease type C. *J. Neurochem.* 136, 74–80 (2015).

31. Ravikumar, B., Moreau, K., Jahrreis, L., Puri, C. & Rubinsztein, D. C. Plasma membrane contributes to the formation of pre-autophagosome structures. *Nat. Cell Biol.* 12, 747–757 (2010).

32. Klionsky, D. J. et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 8, 445–544 (2012).

33. Pirruccello, M. et al. Identification of inhibitors of inositol 5-phosphates through multiple screening strategies. *ACS Chem. Biol.* 9, 1359–1368 (2014).

34. Zhang, X., Li, X. & Xu, H. Phosphoinositide isoforms determine compartment-specific ion channel activity. *Proc. Natl Acad. Sci. USA* 109, 11384–11389 (2012).

35. Verkerke-Mattucci, M., Connelly, P. S., Daniels, M. P. & Puertollano, R. Autophagic dysfunction in mucolipidosis type IV patients. *Hum. Mol. Genet.* 17, 2723–2737 (2008).

36. Abe, K. & Puertollano, R. Role of TRP channels in the regulation of the endosomal pathway. *Physiology (Bethesda)* 26, 14–22 (2011).

37. Grimm, C. et al. Small molecule activators of TRPML3. *Chem. Biol.* 17, 135–148 (2010).

38. Xu, H. & Ren, D. Lysosomal physiology. *Annu. Rev. Physiol.* 77, 57–80 (2015).

39. Nikoletopoulou, V., Papandreou, M. E. & Tavernarakis, N. Autophagy in the physiology and pathology of the central nervous system. *Cell Death Differ.* 22, 398–407 (2015).

40. Di Fusco, G. et al. Lysoplex: an efficient toolkit to detect DNA sequence variations in the autophagy-lysosomal pathway. *Autophagy* 11, 928–938 (2015).
METHODS

Antibodies, cDNAs and reagents. All chemical reagents were of analytical grade or higher and purchased from Sigma-Aldrich unless otherwise specified. The anti-OCRL, -P[48]/K[39]I[31] and -GST polyclonal antibodies have been described in ref. 52. Secondary and secondary antibodies used in this study are listed in Supplementary Table 3. Other reagents: Quant-iT PicoGreen, FuraRed, Hanks' balanced salt solution with calcium and magnesium (HBSS) 1×, Opti-MEM (Life Technologies); SF-51, YU14627(6) (STK016464 and STK883328, respectively) (Vitas-M Laboratory); agaro-se linked anti-GFP antibody (Vector Laboratories); bafilomycin, vinblastine, FCCP, ethidium bromide, uridine, Protein A/G Sepharose Sigma-Aldrich; sodium pyruvate (Euroclone); ODN 2216, ODN 2395, ODN TPAGeoG (Euroclone); Trans-1 (Torcs Bioscience); Tat-becin 1 peptide, digoxigenin (Merck-Millipore). The GST-tagged PH domain of PLC8 was prepared as described previously53. Tandem fluorescent mRFP-eGFP-LC3 was provided by A. Fraidl (TIGEM, Pozzuoli, Italy). TLR9-YFP (plasmid no. 13642) and TIRAP-GFP (plasmid no. 52379) were from Addgene. The full-length WT and mutant form (V527D) of OCRL were prepared previously54.

Mutations in the clathrin-binding domains (LIDIA, 17A and LIDILE, deltaLIDLE) and in the AP2-binding motif (FxDxF; F151S) of OCRL were generated using the QuikChange mutagenesis system (Agilent Technologies) following the manufacturer's protocol using the primers listed in Supplementary Table 4. The GFP-OCRL-X36 triple mutant was generated by sequential mutagenesis of LIDIA, LIDILE and FdxDx domains. Full-length INPP5B (NM_005540) was obtained from HeLa cell cDNA using the primers reported in Supplementary Table 4 and cloned using the restriction sites Sall/Sma1 into the pEGFP-C1 expression vector (Clontech). Mys-DDK-MCOLN1 (NM_020533) was purchased from Origene Technologies. The MCOLN1[R42A;R43A;R44A] mutant (reported in the main text and figures as MCOLN1-3A) was generated using the primers reported in Supplementary Table 4.

Cell culture, transfection and treatments. HK-2 cells were grown as previously described55. HK-2 cells were transiently transfected using TransIT-LT1 (Mirus Bio LLC), unless otherwise specified, according to the manufacturer's instructions, and incubated for 18–24 h before fixation. The HK-2 cell line was checked for mycoplasma contamination by a PCR-based method. The HK-2 cell line was not found in the database of cell lines that are currently known to be cross-contaminated or misidentified by ICLAC and NCBI Biosample. The cell lines used in this study were not authenticated. All cell-based experiments were repeated at least three times.

Proximal tubular cells from Lowe syndrome patients. The procedure for proximal tubular cell (PTC) isolation and immortalization from the urine of control healthy subjects and from Lowe syndrome patients is reported in ref. 5. PTCs were transfected using JetPEI (Polyplus transfection) according to the manufacturer's instructions.

siRNA treatment. siRNA sequences used in this study are listed in Supplementary Table 1. siRNAs against TFEB, described in ref. 8, were provided by C. Settembre (TIGEM, Pozzuoli, Italy).

HK-2 cells were transfected with siRNAs for 96 h using DharmaFECT 4 (Dharmacon) according to the manufacturer's instructions. The siRNA duplexes were used at 50 pmol for OCRL, TLRs, TIRAP, IRAK4, MyD88, DNASel1, UNC93B1 and TFAF and at 25 pmol for PIPSks, MCOLN1, VPS16, STX17, RAB7 and TIRFEB. Mock-treated or non-targeting siRNA-treated HK-2 cells are referred to as controls (CTRL). OCRL-KD was performed using a pool of two OCRL siRNAs from Sigma (N1, N5, used for all experiments unless otherwise stated) and a pool of three OCRL primers reported in Supplementary Table 4.

Quantitative real-time PCR. Real-time quantitative PCR (qRT-PCR) was carried out with the LightCycler 480 SYBR Green 1 mix (Roche) using the Light Cycler 480 II detection system (Roche) with the following conditions: 95 °C, 10 min; (95 °C, 10 s; 60 °C, 10 s; 72 °C, 15 s) × 45 cycles. For expression studies the qRT-PCR results were normalized against an internal control (β-actin and HPRT1).

For mitochondrial gene expression analysis and for the evaluation of mtDNA depletion, qRT-PCR was performed as previously described56. The sequences of primers used in this study are listed in Supplementary Table 4.

Microarray experiments. The Affymetrix Gene-Chip (HG-U133A) hybridization experiments were performed in triplicate at the Correll Genotyping and Microarray Center, Coriell Institute for Medical Research, Camden, New Jersey, USA, on total RNA extracted from control and OCRL-depleted cells. To identify downstream transcriptional effects of OCRL-siRNA treatment, microarray data were pre-processed using the Bioconductor package Affy57 and normalized with the RMA method58. Differentially expressed genes between conditions (KD versus control) were identified using a Bayesian t-test59. For each P value, the Benjamini–Hochberg procedure was used to calculate the false discovery rate (FDR) to avoid the problem of multiple testing.

Gene set enrichment analysis was performed using the freely available software GSEA v2.0 from the Broad Institute60. The goal of GSEA is to determine whether members of a given gene set tend to occur toward the top (or bottom) of a ranked gene list. To run the GSEA algorithm, RMA-normalized microarray data were used. The GSEA algorithm collapsed the probe sets into gene symbols (~13,300 genes) and ranked the genes in the OCRL-KD expression data set on the basis of the fold change after OCRL-KD. GSEA analysis for subcellular distribution was conducted using the Human Protein Atlas for all but lysosomal genes, which were defined according to lysosome61 and excluded from the other categories. To evaluate the significance of the enrichment score the hypergeometric distribution was calculated that describes the probability of k successes in n draws (up- and downregulated genes for each enriched category).

CLEAR analysis. We selected the genes corresponding to the top 250 upregulated probes in the OCRL-KD expression profile. The proximal promoter sequences (1,000 bp upstream and 200 downstream of the transcriptional start site) for each gene were obtained from the UCSC genome browser59 and searched with the matchPWM function in the Bioconductor package Biostrings62 for matches with the CLEAR motif63.

Mitochondrial DNA (mtDNA) depletion and visualization. Mitochondrial DNA depletion was achieved by siRNA knockdown of TFAM or by incubating HK-2 cells in complete medium with ethidium bromide (100 mg ml−1), uridine (50 mg ml−1) and sodium pyruvate (110 mg ml−1) for 4 days to reduce mtDNA levels. Evaluation of depletion was performed using Quant-iT PiCoGreen and qRT-PCR as previously described51,64.

Immune precipitation and western blot. HK-2 cells were plated in 150 mm plates until they reached 95% confluency. Cells were then washed three times with ice-cold 1× PBS, scraped, and proteins were extracted in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA and 1% NP-40 supplemented with phosphatase and protease inhibitor cocktail, Roche), centrifuged for 15 min at 16,000g at 4 °C, and the supernatants were collected. Cell supernatants (1 mg) were incubated for 16 h at 4 °C with 1 μg of the appropriate antibody or with 15 μl of agarose-linked anti-GFP antibody (for TLR9-YFP immunoprecipitation). Samples were then incubated with Protein A/G Sepharose for 2 h at 4 °C except for TLR9–YFP immunoprecipitation. Immunoprecipitates were collected by centrifugation at 5,000 × g at 4 °C, washed five times in lysis buffer and twice in lysis buffer without NP-40 and the proteins were eluted with Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue) and denatured at 95 °C for 5 min. Samples were analysed by SDS–PAGE (8%) under reducing conditions and transferred to nitrocellulose. The membranes were then incubated with the appropriate antibodies. Enhanced chemiluminescence reagent (Euroclone) was used for protein detection. Western blot quantitative analysis (p62/β-actin and LC3/β-actin ratio and p-56K/p56K ratio) was performed using ImageJ band analysis65.

Confocal fluorescence microscopy, super-resolution microscopy, image processing, and co-localization analysis. HK-2 cells and PTCs were grown on glass coverslips and immunofluorescence microscopy and quantitative image analysis were performed as described previously66, except for OCR1–LAMP1 co-labelling where a different permeabilization step was used (that is, 5 min with 20 μl digitonin in buffer A (20 mM Pipes pH 6.8, 137 mM NaCl, 2.7 mM KCl)). The experiments were repeated at least three times and representative images are shown. The level of co-localization (that is, LAMP1–OCRL, LAMP1–TLR9, LAMP1–TIRAP, LAMP1–LC3, LAMP1–AP2, LAMP1–clathrin, LAMP1–TLR9–YFP, LAMP1–TIRAP, LAMP1–(P4/K39/I31), LAMP1–PI5K1α, LAMP1–PI5K1β, LAMP1–PI(4,5)P2, MCOLN1–OCRL, MCOLN1–LAMP1) was calculated by acquiring confocal serial sections from about 50–100 cells per experimental condition per experiment, exported in TIFF format, and processed as previously described66. Briefly confocal images of at least 50–100 cells per condition were acquired at the same laser power and photomultiplier gain. Images were then processed using ImageJ software. Single channels from each image were converted into 8-bit greyscale images and thresholded to subtract background. The Image 'Analyze Particles' plugin was then used to identify and count the total number of the structures (with an area above 0.10 μm2) in channel 1 (that is, LAMP1). The structures in channel 2 (that is, OCRL) were used to build a mask that was then overlapped with the structures containing both markers. The remaining structures, positive only for LAMP1, were counted and difference the number of structures containing both LAMP1 and OCRL was calculated and expressed as a percentage of the total number of

DOI: 10.1038/ncb3386
**METHODS**

LAMP1-positive structures. Note that this quantitative analysis procedure does not use merged images and is not affected by the fluorescence intensity.

For conventional confocal microscopy a confocal laser microscope (Zeiss LSM 700 confocal microscope systems; Carl Zeiss) with a ×63 1.4 NA oil immersion objective was used. Super-resolution microscopy was performed with a Zeiss LSM 880 (Carl Zeiss). Airyscan-equipped confocal microscope with a ×63 1.4 NA oil immersion objective. After acquisition, images were processed with the Airyscan processing tool available on the Zen software provided by Zeiss, with a processing threshold set at 3.9.

**PdIns(4,5)P2 detection.** Control (mock) and OCRL-siRNA-treated HK-2 cells were grown to 70–80% density on glass coverslips and PdIns(4,5)P2 staining was performed according to ref. 60. Briefly, cells were fixed for 15 min at room temperature by addition of 1 volume of pre-warmed 4% PFA (paraformaldehyde in PBS) to the growth medium (2% PFA final concentration). Cells were then permeabilized for 5 min with 20 μM digitonin in buffer A. Cells were then blocked for 45–60 min with buffer A containing 5% PBS (fetal bovine serum) and 50 μg/mL bovine serum albumin (BSA) and 0.1 M sodium citrate in distilled water, pH 6.0, and 0.1 M citric acid. Before staining, slides were deparaffinized in xylene for 5 min, washed once in milliQ water and analysed using a confocal laser microscope (Zeiss LSM 700 confocal microscope systems; Carl Zeiss) with a ×63 1.4 NA oil immersion objective. Quantitative analysis of PdIns(4,5)P2–LAMP1 co-localization was performed as previously reported1.

**Autophagy induction by Torin 1 and Tat-beclin 1 peptide.** HK-2 cells were treated for 2 h with 250 nM of the specific mTOR inhibitor Torin 1 (Tocris Bioscience) to induce autophagy. Cells were washed twice with PBS and then incubated with Opti-MEM and then incubated for 2 h with 20 μM of the Tat-beclin 1 peptide2,2 dissolved in acetylated Opti-MEM (9 mM HCl).

**Quantitative analysis of LC3 structures.** CellProfiler, a free, open-source image analysis software (www.cellprofiler.org), was used for quantitative analysis of confocal microscopy images by applying specific pipelines described in ref. 61. The pipeline ‘Speckle Counting’ was used to identify smaller objects (LC3-positive structures) surrounding larger objects (nuclei) and to perform per-object aggregate measurements (such as the number of LC3-positive structures per nucleus). Briefly, the images were converted to greyscale using the module ‘ColorToGray’. Then the ‘ObjectIdentification’ module was used to identify nuclei (called primary objects) on the basis of Hoechst staining. Secondary objects (LC3-positive structures) were detected and counted using the module ‘IdentifySecondaryObjects’ by expanding the area surrounding the primary object by 20 pixels.

**Human kidney biopsies.** Human kidney biopsies were obtained from archived samples of three patients with clinical diagnosis of Lowe syndrome, corroborated in one case (shown in Fig. 6f) with the genetic diagnosis of mutation in OCRL, two patients affected by glomerular (and not tubular) disease, and two controls (non-transplant patients) of the 12 patients. The procedure was approved by the Ethical Review Board of Saint-Luc Academic Hospital (Brussels, Belgium) and the EURenOmics consortium (FP7, 2007–2013, grant agreement no. 305608).

**Immunofluorescence on human kidney samples.** Paraffin blocks of human kidney samples were sectioned into consecutive slices with a thickness of 6 μm using a Leica RM2235 rotary microtome (Thermo-Fisher Scientific) on Superfrost Plus glass slides (12–550–15, Thermo-Fisher Scientific). Before staining, cells were deparaffinized in changes of CitriSolv (22-143-975, Thermo-Fisher Scientific.) and 70% isopropanol. Antigen retrieval was accomplished by incubating in sodium citrate buffer (1.8% 0.1 M citric acid, 8.2% 0.1 M sodium citrate, in distilled water, pH 6.0) in a rice cooker for 30 min. The slides were blocked with PBS blocking buffer (1% BSA, 0.2% non-fat dry milk in PBS) for 30 min at room temperature. Anti-LC3B primary antibodies were used to represent changes in cytosolic Ca2+ levels in conditional knockout (null) YU142670) or 0.5 μM of TLR9 antagonist (ODN TTAGGG). Where reported, cells were also treated with protease inhibitor cocktail (PI: leupeptin, E64D and pepstatin, Sigma-Aldrich) for the indicated time points. After fixation with 4% PFA, cells were washed three times with 1× PBS containing 50 μM NH4Cl, mounted and imaged using a Zeiss LSM 710 confocal microscope (equipped with a ×63 1.4 NA oil immersion objective).

**Calcium measurements by confocal imaging using FuraRed.** FuraRed-loaded cells were analysed according to the previously described protocol14. Briefly, mock HK-2 cells or OCRL-silenced HK-2 cells were loaded with 5 μM FuraRed-AM for 1 h at 37 °C in calcium imaging buffer. Pseudocolour ratioimetric images (458 and 488 nm excitation, 610 nm emission) were collected using a Leica Zeiss microscope (LSM 710) equipped with a variable filter wheel. FuraRed ratios (emission F485/F405 nm) were used to represent changes in cytosolic Ca2+ levels in basal conditions or after specific treatments. The number of cells that mobilized calcium was quantified in a minimum of three wells and compared with control cells transfected with scramble siRNAs. More than 20 cells in triplicate were used in the different experiments.

**Statistics and reproducibility.** For experiments involving living animals no statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were blinded to allocation during experiments and outcome assessment.

Immunofluorescence experiments were repeated independently three times and at least 50 cells were analysed from a single experiment. Representative images are shown in Figs 1c,d,e, 2a,c, 3a, 4a,b,d,g, 5a,d,e, 6a,d,f,g and 7a,e,f and in Supplementary Figs 2b, 3b, d, 4b, d, e, g, 5a, c, e, f, 6a, b and 7a.

Zebrifish strains and husbandry. Zebrifish were maintained at The University of Manchester Biological Services Unit in accordance with the policies of the UK Animal (Scientific Procedures) Act 1986. Wild-type adult and larval wild-type and mutants were obtained from the National Zebrafish Resource Centre, University of Edinburgh, UK. Fertilized eggs were allowed to develop until the 1-2 day stage. At this stage, mutants and wild-type siblings were selected. Mutants were identified as 2,4,6-trinitrophenyl (TNP) positive (red) using a Nikon Model Ds1 digital camera (Nikon, UK) and the animal experiments were performed in accordance with the UK Home Office Animal Act 1986. Wild-type and mutant siblings were selected at this time point and used for all experiments. The ocrl1 mutant line (ZDB-GENO-120531-1) was previously described20,21.

The only procedure involving animals was the breeding of transgenics, which is subject to local ethical review and performed under a Home Office licence. No statistical method was used to predetermine sample size; the experiments were not randomized, and the Investigators were not blinded to allocation during experiments and outcome assessment. Experiments were performed on larvae at 3 days post-fertilization, at which point they are not classified as animal experiments.

**Electron microscopy.** Cells for pre-embedding immunoelectron microscopy were fixed, permeabilized, and labelled as described previously39. From each sample, thin 65 nm sections were cut using a Leica EM UC7 ultramicrotome. EM images were acquired from thin sections using an FEI Tecnai-12 electron microscope (FEI) equipped with a VILTEA CCD (charge-coupled device) digital camera (Soft Imaging Systems GmbH). We used the following criteria for morphological identification: lysosomes (Ly): LAMP1-positive single-membrane-bound bodies containing electron-dense material; multivesicular bodies (MVB): LAMP1-positive single-membrane-bound bodies containing more than nine intralumenal vesicles; multivesicular bodies (MVB): LAMP1-positive single-membrane-bound bodies containing myelin-like figures; autophagic vacuoles (AV): double-membrane-bound vacuoles containing sequestered material (only cytoplasm, cytoplasm and/or ER, cytoplasm and/or mitochondria, ER and/or mitochondria); autolysosomes (AL): LAMP1-positive single-membrane-bound bodies containing electron-dense cytoplasmatic material and/or organelles. The number of autophagosomes was then calculated using iTEM software (Olympus SYS). Zebrifish samples were processed for electron microscopy as described previously3.

**Dissection of autophagosome–lysosome fusion by tandem fluorescent LC3.** Control (mock), OCRL- and TLR9- siRNA-treated HK-2 cells were grown to 70% confluency and transiently transfected with a plasmid encoding an mRFP-GFP-tagged LC32. After transfection, cells were kept in growth medium for 16 h and then incubation was continued in normal growth medium or the cells were treated with HBSS for 3 h. CTRL cells were also treated for 3 h with 25 μM of OCRL inhibitor (YU142670) or 0.5 μM of TLR9 antagonist (ODN TTAGGG). Where reported, cells were also treated with protease inhibitor cocktail (PI: leupeptin, E64D and pepstatin, Sigma-Aldrich) for the indicated time points. After fixation with 4% PFA, cells were washed three times with 1× PBS containing 50 μM NH4Cl, mounted and imaged using a Zeiss LSM 710 confocal microscope (equipped with a ×63 1.4 NA oil immersion objective).
Most data are presented as the means ± standard deviation (s.d.) unless otherwise specified. Statistical comparisons were made using Student’s t-test when comparing two groups. One-way analysis of variance (ANOVA) with Tukey’s post hoc test was performed for comparisons among more than two groups relative to a single factor (treatment or siRNA-induced knockdown). Two-way ANOVA and Tukey’s post hoc test was performed for comparisons among more than two groups relative to two factors (treatment and siRNA-induced knockdown). These analyses were performed in the R (ref. 65) (http://www.R-project.org) environment. For all analyses, a P value < 0.05 was considered to be statistically significant.

For the quantification of LC3- and LAMP1-positive structures in kidney biopsies, ANOVA was also used. A P value < 0.05 was considered to be statistically significant.

Data availability. Microarray data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE54284. Source data for Figs 2b,c, 3a,c, 4a,c,e,f,h, 5b–g, 6b–e,g and 8b,c,d, and for Supplementary Figs 2b, 3a,b,e, 4e,f,g, 5a,f and 7b are available in Supplementary Table 2: Statistic source data. All other data supporting the findings of this study are available from the corresponding author on request.

51. Yuan, Y. et al. Mitochondrial dysfunction accounts for aldosterone-induced epithelial-to-mesenchymal transition of renal proximal tubular epithelial cells. Free Radic. Biol. Med. 53, 30–43 (2012).

52. Gautier, L., Cope, L., Bolstad, B. M. & Irizarry, R. A. affy–analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20, 307–315 (2004).

53. Irizarry, R. A. et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264 (2003).

54. Baldi, P. & Long, A. D. A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. Bioinformatics 17, 509–519 (2001).

55. Kuhn, R. M., Haussler, D. & Kent, W. J. The UCSC genome browser and associated tools. Brief. Bioinform. 14, 144–161 (2013).

56. Wasserman, W. W. & Sandelin, A. Applied bioinformatics for the identification of regulatory elements. Nat. Rev. Genet. 5, 276–287 (2004).

57. Sardiello, M. et al. A gene network regulating lysosomal biogenesis and function. Science 325, 473–477 (2009).

58. Ashley, N., Harris, D. & Poulton, J. Detection of mitochondrial DNA depletion in living human cells using PicoGreen staining. Exp. Cell Res. 303, 432–446 (2005).

59. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675 (2012).

60. Hammond, G. R., Schiavo, G. & Irvine, R. F. Immunocytochemical techniques reveal multiple, distinct cellular pools of PtdIns4P and PtdIns(4,5)P2. Biochem. J. 422, 23–35 (2009).

61. Ladoire, S. et al. Immunohistochemical detection of cytoplasmic LC3 puncta in human cancer specimens. Autophagy 8, 1175–1184 (2012).

62. Luciani, A. et al. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. Nat. Cell Biol. 12, 863–875 (2010).

63. R_Core_Team R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, 2014).
Erratum: Autophagosome–lysosome fusion triggers a lysosomal response mediated by TLR9 and controlled by OCRL

Maria Giovanna De Leo, Leopoldo Staiano, Mariella Vicinanza, Alessandro Luciani, Annamaria Carissimo, Margherita Mutarelli, Antonella Di Campli, Elena Polishchuk, Giuseppe Di Tullio, Valentina Morra, Elena Levchenko, Francesca Oltrabella, Tobias Starborg, Michele Santoro, Diego di Bernardo, Olivier Devuyst, Martin Lowe, Diego L. Medina, Andrea Ballabio and Maria Antonietta De Matteis

Nature Cell Biology 18, 339–850 (2016); published online 11 July 2016; corrected online 18 July 2016

In the version of this Article originally published online, in Fig. 1e, the ‘WT’ label should have been in the top panel, not the second panel. This has been corrected in all versions of the Article.
Supplementary Figure 1 Effects of siRNA treatment on protein and mRNA levels. (a) HK-2 cells were treated with non-targeting siRNA (CTRL) or with Sigma Aldrich (S) or Ambion (A) OCRL siRNA pools for 96 hours. Cell lysates (50 μg/sample) were analyzed by SDS-PAGE and immunoblotted using an anti-OCRL antibody or an anti-β-actin antibody as a loading control. A representative blot is shown. An unprocessed scan of the blot is shown in Supplementary Fig. 9. The graph reports the results of the quantitative analysis of OCRL levels from n=10 independent experiments for CTRL (mock) and OCRLKD (S) and n=5 independent experiments for CTRL (non-targeting) and OCRLKD (A). The data are expressed as the level of OCRL normalized for β-actin as a % of the CTRL. Mean values ± s.d. (b) Representative images of CTRL (non-targeting siRNA) and OCRL-KD HK-2 cells stained for OCRL, TGN46 (a Trans Golgi Network marker) and EEA1 (an Early Endosome marker). Insets represent OCRL-TGN46 and OCRL-EEA1 merged enlargements of the boxed areas. Scale bars, 10 mm. (c) HK-2 cells were treated with the indicated siRNAs (Listed in Supplementary Table 1). Total RNA (1 μg/sample) was analyzed by qRT–PCR using specific primers (Listed in Supplementary Table 4). Expression levels in siRNA-treated cells are reported relative to the expression of the corresponding gene in mock-treated cells (CTRL) normalized to β-actin. All qPCR reactions were carried out in triplicate and the data represent mean values ± s.d. from n=5 independent experiments. Statistical significance calculated by Student's t-test.
Supplementary Figure 2 OCRL depletion induces upregulation of lysosomal genes and TFEB nuclear translocation. (a) Fold change expression of the indicated lysosomal genes (selected amongst those most upregulated in the microarray) in OCRL-KD (Ambion siRNAs pool) cells relative to non-targeting siRNA-treated cells (CTRL) as assessed by quantitative RT-PCR carried out in triplicate and the data represent mean values ± s.d. from n=6 independent experiments. p values calculated using Student’s t test (unpaired). (b) CTRL, OCRL-KD (treated either with Sigma (S) or Ambion (A) pools) and YU142670-treated HK-2 cells were stained with an anti-TFEB antibody (green). Mean values ± s.d. n=150 cells pooled from 3 independent experiments. Numbers on the images report the percentage of cells with nuclear TFEB. p < 1.6x10⁻⁷ for CTRL vs. OCRL-KD (S), OCRL-KD (A) and YU142670-treated cells calculated by One-way ANOVA with Tukey’s post-hoc test. Scale bars, 10 µm. Statistic source data for Supplementary Fig. 2b can be found in Supplementary Table 2. (c) The position of the TFEB binding site (CLEAR motif, blue boxes) relative to the transcriptional start site (0) in the promoters of the indicated upregulated lysosomal genes in OCRL-KD cells, assessed as described in Methods. The color intensity is proportional to the match score (the darker the higher). (d) Residual upregulation of the indicated lysosomal genes in HK-2 cells knocked down for both OCRL and TFEB relative to OCRL-KD cells as assessed by quantitative RT-PCR carried out in triplicate. The data represent mean values ± s.d. from n=6 independent experiments. p values were calculated by Student’s t-test. NS, not significant. Notice the inverse correlation between the presence of a CLEAR motif and the extent of residual upregulation upon TFEB depletion.
Supplementary Figure 3  OCRL, AP2 and clathrin recruitment to lysosomes during autophagy requires the synthesis of PI(4,5)P$_2$ and is independent of mTOR activation or inactivation.  (a) Representative image of the colocalization of PI4KIII$\beta$ with LAMP1 in HBSS. Scale bar, 10 mm. The graph reports quantification of PI4KIII$\beta$ with LAMP1 colocalization in growth medium and HBSS. Mean values ± s.d. n=200 cells pooled from 3 independent experiments. (b) Quantification of PI(4,5)P$_2$, AP2, clathrin and OCRL colocalization with LAMP1 during starvation (HBSS for 3 hours) after impairing PI(4,5)P$_2$ synthesis (PI4KIII$\beta$-KD, PIP5K1$\alpha$-KD and PIP5K1$\beta$-KD). Mean values ± s.d. n=200 cells per condition pooled from 3 independent experiments. (c) Cell lysates (50 mg) from HK-2 cells treated with HBSS and then re-fed by addition of complete growth medium for the indicated time points were analyzed by SDS-PAGE and immunoblotted using an anti-p70, an anti-phospho-p70 (p-p70), or an anti-GAPDH antibody as a loading control. A representative blot is shown and an unprocessed scan of the blot is shown in Supplementary Fig. 9. (d) Starvation-independent autophagy stimuli induce an increase in PI(4,5)P$_2$ and recruitment of AP2, clathrin and OCRL to lysosomes. HK-2 cells were treated with the mTOR inhibitor Torin-1, with Tat-beclin 1 peptide (see Methods), or left untreated (Growth medium) and then stained for OCRL and LAMP1. The insets are enlargements of the boxed areas. Scale bars, 10 mm. (e) Quantification of PI(4,5)P$_2$, AP2, clathrin and OCRL colocalization with LAMP1 and of the average number of autophagosomes (LC3 structures) per cell in untreated cells and in cells treated with Torin-1 and Tat-beclin 1 peptide. Mean values ± s.d. n=100 cells per condition pooled from 3 independent experiments. p values calculated by one-way ANOVA with Tukey’s post-hoc test. Statistic source data for Supplementary Fig. 3 can be found in Supplementary Table 2.
Supplementary Figure 4: Inhibiting TLR9 or depleting mtDNA inhibits the lysosomal recruitment of OCRL and impairs the recycling of the autophagosomal SNARE STX17. (a) IEM of fed or starved TLR9-YFP expressing HK-2 cells and stained with an anti-GFP antibody (scale bar, 200 nm). Arrows, TLR9 in the ER (white), in Multivesicular bodies/Lysosomes (black), or in autolysosomes (red). (b) Control, TFAM-KD and EtBr treated HK-2 cells stained with PicoGreen to visualize mitochondrial DNA. Scale bars, 10 μm. (c) Expression of the indicated nuclear (α-SMA) and mitochondrial (ND-6, D-loop, CoxI and CoxII) genes evaluated by qPCR in EtBr-treated cells vs. control cells. All qPCR reactions were carried out in triplicate and the data represent mean values ± s.d. from n=3 independent experiments. (d) OCRL (red) association with lysosomes upon starvation in CTRL, TFAM-KD, and HK-2 cells treated with EtBr. Scale bars, 10 μm. Insets, enlargements of the boxed areas. (e) Fed or starved HK-2 cells expressing TIRAP-GFP stained for LAMP1. Insets, enlargements of the boxed areas. Scale bars, 10 μm. Graph, colocalization of TIRAP with LAMP1. Mean ± s.d. n=100 cells per condition pooled from 3 independent experiments. (f) Fed or starved HK-2 cells KD for adaptors (MyD88 and TIRAP) or transducers (IRAK4) of TLR9 signalling and for UNC93B1. LAMP1-OCRL colocalization. Mean values ± s.d. n=300 cells per condition pooled from 3 independent experiments. (g) Representative images of control or TLR9-KD HK-2 cells expressing the STX17-GFP incubated in HBSS for 3 hours. Insets, enlargements of the boxed areas. Scale bars, 10 μm. Right graph, quantification of LAMP1-STX17 colocalization. n=75 cells per condition pooled from 3 independent experiments. The data show that STX17 is enriched in LAMP1-positive compartments in TLR9-depleted cells as compared to control cells. The LAMP1 structures containing STX17 are, by definition, autolysosomes: since the total number of autolysosomes is lower in TLR9-KD cells (Figure 5b, e), then the fraction of autolysosomes still containing STX17 is markedly increased upon TLR9 deletion, suggesting that recycling of STX17 from autolysosomes is under control of TLR9. The p values are indicated, calculated using Student’s t-test in c, e, and by one-way ANOVA with Tukey’s post-hoc test in f, g. Statistic source data in Supplementary Table 2.

© 2016 Macmillan Publishers Limited. All rights reserved.
Supplementary Figure 5 HK-2 cells depleted of OCRL accumulate mature autophagosomes following impairment of autophagosome/lysosome fusion. (a) Representative images of CTRL and OCRL-KD cells incubated in growth medium or in HBSS for 3 hours, stained for ATG16L1 (an early and transient autophagosomal marker). Scale bars, 10 µm. The graph reports the quantification of the ATG16L1-positive structures, means ± s.d. n=150 cells pooled from 3 independent experiments. (b) Immunoblot analysis of cell lysates (50 µg/sample) from CTRL, OCRL-KD (Sigma (S) or Ambion (A) siRNA pools) and YU142670-treated cells with or without 100 nM Bafilomycin A1 (Baf A1) for 2 hours with the indicated antibodies. β-actin was used as a loading control. A representative blot is shown and an unprocessed scan of the blot is shown in Supplementary Fig. 9. Mean values ± s.d. n=3 lysates per condition pooled from 3 independent experiments. (c) HK2 cells were depleted of OCRL using Ambion siRNA pool and treated as described in Figure 6d. Mean values ± s.d. n=120 cells per condition pooled from 3 independent experiments. (d) HK2 cells were depleted of OCRL using Ambion siRNA pool and treated as described in Figure 6d. The arrow indicates an autolysosome. Mean values ± s.d. n=150 cells per condition pooled from 3 independent experiments. (e) CTRL and OCRL-KD cells were incubated in HBSS for 3 hours and labeled with an anti-LAMP1 (green) antibody together with an anti-GST antibody to detect recombinant GST-PHPLCδ (a probe for PI(4,5)P₂). White arrows indicate structures positive for both markers. Scale bars, 10 µm. (f) Representative images and quantification of PI(4,5)P₂-LAMP1 colocalization in OCRL-KD cells expressing an siRNA-resistant wild-type (GFP-OCRL wt) or catalytically inactive (GFP-OCRL V527D) OCRL and stained with antibodies against PI(4,5)P₂ (red) and LAMP1 (green). Transfected cells are outlined. Scale bars, 10 µm. Mean values ± s.d. n=300 cells pooled from 3 independent experiments. p values are indicated, calculated by One-way ANOVA with Tukey’s post-hoc test in a, c, d, f, and by Student’s t-test in b; NS (not significant). Statistic source data for Supplementary Fig. 5 can be found in Supplementary Table 2.
**Supplementary Figure 6** Accumulation of autophagosomes and expansion of lysosomal compartments in kidneys of Lowe syndrome patients. (a) Representative confocal micrographs of proximal tubules from two patients with Lowe syndrome and one control immunostained with an anti-LC3 (red) and anti-AQP1 (green, a proximal tubule marker) antibody. Quantification of the LC3-positive structures is given in Figure 6f. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μm. (b) Representative confocal micrographs of proximal tubules from two patients with Lowe syndrome and one control immunostained with an anti-LAMP1 (red) and anti-AQP1 (green) antibody. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μm. The graph reports the quantification of LAMP1-positive structures in AQP1-positive proximal tubules in human kidney biopsies from three Lowe syndrome patients and four controls. Data are means ± s.e.m., n=50 proximal tubules pooled from 10 fields per biopsy. *p < 0.0025 calculated using one-way ANOVA/post-hoc tests.
Supplementary Figure 7 The MCOLN1 agonist SF-51 specifically reverts autophagosome accumulation in OCRL-depleted cells and in PTCs from Lowe syndrome patients. (a) OCRL depletion does not affect MCOLN1 localization at lysosomes. Mock-treated (CTRL) and OCRL-KD HK-2 cells transiently expressing MCOLN1-myc were immunostained with anti-myc (green) and anti-LAMP1 (red) antibodies. Numbers indicate the percentage of LAMP1-MCOLN1 colocalization. Means ± s.d., n=150 transfected cells per condition pooled from 3 independent experiments. Scale bar, 10 μm. (b) SF-51 does not rescue the autophagosome accumulation caused by depleting components of the autophagosome-lysosome tethering/fusion machinery. Mock-treated (CTRL), OCRL-KD, MCOLN1-KD, RAB7-KD and VPS16-KD HK-2 cells incubated in growth medium with or without 200 μM of the mucolipin-1 agonist SF-51 for 2 hours were immunostained for LC3 (red) and LAMP1 (green). The graph reports the quantification of the number of LC3-positive structures per cell. Means ± s.d. n=300 cells per condition pooled from 3 independent experiments. p values are indicated calculated by One-way ANOVA with Tukey’s post hoc test. Scale bar, 10 μm.
Supplementary Figure 8 Working model depicting the lysosome cargo response. At steady state OCRL associates with clathrin-coated pits (CCP), clathrin-coated vesicles (CCV), early endosomes and the Trans Golgi Network (TGN) (and only marginally with lysosomes) and the phosphoinositides that are mainly present in lysosomes are PI3P and PI(3,5)P₂. When autophagosomes (AV) fuse with lysosomes (LY) during starvation, the mtDNA released into lysosomes activates TLR9, which translocates from the endoplasmic reticulum (ER) to autolysosomes (AL). TIRAP, a known component of the TLR9 signalling pathway, also translocates to AL. The activation of TLR9 in AL induces the recruitment of PI(4,5)P₂-producing enzymes (PI(3,5)KTα and β) that leads to an increase in PI(4,5)P₂. This increase is restricted by the simultaneous recruitment of OCRL, a PI(4,5)P₂ 5-phosphatase. The lysosomal translocation of OCRL is driven by the clathrin adaptor AP2 and clathrin, which associate with lysosomes in a PI(4,5)P₂-dependent manner. The fine spatial control of PI(4,5)P₂ in lysosomes is required to preserve the activity of MCOLN1 (required for autophagosome-lysosome fusion) which is inhibited by PI(4,5)P₂. OCRL, which interacts with MCOLN1, ensures PI(4,5)P₂-free microdomains around MCOLN1.
Supplementary Figure 9 Unprocessed scans of original blots shown in figures and supplementary figures. The uncropped and unprocessed scans of original blots shown in this study are reported.
Supplementary Table Legends

Supplementary Table 1
siRNA oligonucleotides used in this study.
Names of the genes, RNA sequences, company names and catalog numbers (if available) of the siRNAs used in this study are reported.

Supplementary Table 2
Statistic source data.
The data used to derive the statistics of all the relevant experiments shown in main and supplementary figures are reported.

Supplementary Table 3
Primary and secondary antibodies used in this study.
Names, catalog numbers, clone numbers (only for monoclonal antibodies), company names, working dilution and application in which the antibodies were used in this study are described.

Supplementary Table 4
Sequence of oligonucleotides used in this study.
Names and DNA sequences of qPCR, mutagenesis and cloning primers used in this study are reported.