Proton-gated anion transport governs macropinosome shrinkage

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Intracellular organelles change their size during trafficking and maturation. This requires the transport of ions and water across their membranes. Macropinocytosis, a ubiquitous form of endocytosis of particular importance for immune and cancer cells, generates large vacuoles that can be followed optically. Shrinkage of macrophage macropinosomes depends on TPC-mediated Na\(^+\) efflux and Cl\(^-\) exit through unknown channels. Relieving osmotic pressure facilitates vesicle budding, positioning osmotic shrinkage upstream of vesicular sorting and trafficking. Here we identify the missing macrophage Cl\(^-\) channel as the proton-activated Cl\(^-\) channel ASOR/TMEM206. ASOR activation requires Na\(^+\)-mediated depolarization and luminal acidification by redundant transporters including H\(^+\)-ATPases and CLC 2Cl\(^-\)/H\(^+\) exchangers. As corroborated by mathematical modelling, feedback loops requiring the steep voltage and pH dependencies of ASOR and CLCs render vacuole resolution resilient towards transporter copy numbers. TMEM206 disruption increased albumin-dependent survival of cancer cells. Our work suggests a function for the voltage and pH dependence of ASOR and CLCs, provides a comprehensive model for ion-transport-dependent vacuole maturation and reveals biological roles of ASOR.

With the exception of tubular structures, reduction of vesicle size cannot occur solely by the release of smaller vesicles because vesicle volume depends on the third power, and surface area on the square, of the radius. Although small amounts of luminal fluid might be taken up by tubular endolysosomes in ‘kiss-and-run’ processes, decrease of vesicle volume requires transmembrane water flux that is driven by osmotic gradients. The shrinkage (‘resolution’) of the large vacuoles generated by macropinocytosis is followed in macropinosomes in pulse-chase experiments. Macropinosome (MP) resolution requires luminal Na\(^+\)\(^+\) and Cl\(^-\) (ref. 5). Whereas Na\(^+\) flows through TPC1 and TPC2 cation channels, the channel(s) mediating the required parallel Cl\(^-\) conductance remained unknown. In this Article, using macrophages from mice with disrupted Cl\(^-\) transporter genes, we set out to identify the underlying channel, elucidate its interaction with other vesicular ion transporters in vesicle maturation and study its biological roles.

Results

In bone-marrow-derived macrophages (BMDMs), addition of macrophage colony-stimulating factor (M-CSF) rapidly triggers the formation of MPs, which can be labelled by engulfed dextran-coupled fluorescent dyes. Their initial luminal ion composition closely mirrors that of the extracellular medium. We followed the volume of 70kDa tetramethylrhodamine (TMR)–dextran containing MPs from 5 to 15 min after M-CSF addition in live cell imaging (Fig. 1a,b). MPs shrank to roughly 20% of initial volume during this period (Fig. 1c,d and Supplementary Video 1). Concomitantly, vesicular fluorescence intensity, reflecting luminal TMR–dextran concentration, increased by ~50% (Fig. 1c,d). As described\(^1\), substitution of luminal Na\(^+\) (by N-methyl-d-glucamine) or Cl\(^-\) (by gluconate) strongly impaired both MP resolution and the increase in fluorescence (Fig. 1c,d).

Identification of MP Cl\(^-\) conductance. Several candidates were proposed to mediate the Cl\(^-\) conductance in parallel to TPC Na\(^+\) channels\(^3\). These include CIC 2Cl\(^-\)/H\(^+\) exchangers\(^5\), CLC1\(^-\) and VRAC/LRRC8 anion channels\(^6\) (Fig. 1e). Despite their name, CLCs (CLIntracellular Channels) are unlikely to form physiological Cl\(^-\) channels\(^7\). Volume-regulated anion channels (VRACs), hexamers containing LRRC8A (refs. 8,12) and at least one other LRRC8 isoform\(^13\), reside in the plasma membrane, but contradictory reports\(^14,14\) suggest that VRACs may also regulate lysosomal volume. Because MPs are initially formed by the plasma membrane, we also considered the plasma membrane Cl\(^-\) channel CIC-2 and the ubiquitously expressed acid-activated anion channel ASOR\(^15,16\) (also known as PAORAC\(^18\) or PAC\(^19\)), which were\(^6\) and others\(^14\) have recently identified as being formed by TMEM206 proteins. CIC-2 and CIC-6 were almost absent, CIC-3 and CIC-4 barely detectable, and CIC-5, CIC-7, LRRC8A and TMEM206 robustly expressed in BMDMs (Fig. 1f–m and Extended Data Fig. 1). We investigated the subcellular localization of the most highly expressed candidates, CIC-7 and TMEM206, in BMDMs under resting conditions (Fig. 2a,c,d and Extended Data Fig. 2) and after M-CSF exposure (Fig. 3). As in other cells\(^15,18\), CIC-7 co-localized with lysosomal LAMP1 and partially with late endosomal rab7, but not with early endosomal EE1 (Fig. 2d and Extended Data Fig. 2c). Unlike CIC-7, ASOR/TMEM206 was detected at the plasma membrane (Fig. 2a) where it mediated typical acid-activated, outwardly rectifying Cl\(^-\) currents (Fig. 2b). However, most of TMEM206 was intracellular where it co-localized with EE1 and rab5, but only partially with LAMP1 and rab7 (Fig. 2c and Extended Data Fig. 2a). Confirming a recent report\(^1\), ASOR was found on endosomes also in human embryonic kidney (HEK) cells (Extended Data Fig. 3).

M-CSF rapidly induced the formation of MPs that were early on positive for EE1 and rab5, but not rab7 (Figs. 3a,d at 4 min), which they acquired later (Fig. 3b,e at 7 min). TMEM206, but not CIC-7,
**Fig. 1 | Ions and candidate ion transporters in MP shrinkage.** a, Scheme of experimental approach. b, Fluorescence images of MPs formed in presence of 70 kDa TMR-dextran, 5 and 15 min after M-CSF addition. Scale bars, 5 µm. c, Removal of luminal Na\(^+\) or Cl\(^-\) impairs MP resolution, measured by vesicle volume (left) or TMR-dextran fluorescence (right). \(n=11, N=743\) (WT); \(n=7, N=633\) (low Na\(^+\), near 0 mM); \(n=10, N=869\) (low Cl\(^-\), 9 mM). \(n\) is number of animals, \(N\) is number of MPs. Plot of mean ± standard error of the mean (s.e.m.) (shown as bands), averaging means from individual mice. d, Mean MP volume 15 min after M-CSF normalized to volume at 5 min as function of luminal ion concentrations. Data points, mean values from individual mice. Error bars, s.e.m. One-way ANOVA with Tukey’s multiple comparison shown with regard to NaCl. e, Ion transporter candidates. f–m, Western blot expression analysis of TMEM206 (specific bands indicated by arrows) (f), LRRC8A (g), ClC-2 (h), ClC-3 (i), ClC-4 (j), ClC-5 (k), ClC-6 (l) and ClC-7 (m) in mouse BMDMs, compared with organs highly expressing the respective proteins. α1 Na/K-ATPase (f) and actin (g–m) were used as loading control. \(n=3\) for each western blot. Source numerical data and unprocessed blots are available in source data.
was detected on MPs during the first ~10 min (Fig. 3a–e). Later (Fig. 3f), MPs acquired ClC-7, probably by fusion with lysosomes. ASOR trafficking was followed by live cell imaging of BMDMs transfected with fluorescently tagged TMEM206 (Fig. 3c) that localized similar to the native protein (Extended Data Fig. 2a,b). MPs formed by applying M-CSF together with TMR–dextran showed red luminal fluorescence surrounded by green ASOR-GFP (green fluorescent protein) (Fig. 3c and Supplementary Video 2). They shrank over time and concentrated TMR–dextran in their lumina. Hence, ASOR/TMEM206 emerged as an excellent candidate for the Cl\(^--\) channel involved in MP shrinkage.

We collected BMDMs from Tmem206\(^{-/-}\) mouse lines (Extended Data Fig. 4a–d), which lacked obvious pathology, and from Cx3cr1-Cre\(^{ER\text{2}2}\), Clcn7\(^{lox/lox}\) mice in which Clcn7 was disrupted in the monocyte lineage to avoid the osteopetrosis and early death of Clcn7\(^{-/-}\) mice\(^{22}\). Whereas Clcn7 disruption had no effect (Extended Data Fig. 5d), ASOR deletion impaired shrinkage to the same degree as luminal Cl\(^--\) replacement (Fig. 4a–c and Supplementary Videos 1 and 3). In human HT-1080 cancer cells\(^{23}\), an 80% reduction of TMEM206 protein levels by short interfering RNA (siRNA) (Extended Data Fig. 4e) sufficed to slow the resolution of MPs that were generated in response to epidermal growth factor (EGF) (Fig. 4d–f). Off-target effects were excluded by using three Tmem206\(^{-/-}\) mouse lines generated with different single guide RNAs (sgRNAs) (Extended Data Fig. 4d) and by rescuing MP resolution of Tmem206\(^{-/-}\) BMDMs by transfecting TMEM206 (Fig. 4g). Overexpression of TMEM206 failed to significantly accelerate MP resolution beyond wild type (WT) levels in both Tmem206\(^{-/-}\) and WT BMDMs. Rescue of resolution depended on ASOR’s ion conductance because the transport-deficient K319C mutant\(^{18}\) had no significant effect (Fig. 4g). Rescue of MP resolution did not require an N-terminal tyrosine motif that enhances endosomal targeting\(^{21}\).
(Fig. 4b). Disruption of Tmem206 or ion replacements did not affect acute, M-CSF-triggered formation of MPs of BMDMs (Extended Data Fig. 6a). This is expected from ASOR’s role in luminal salt loss and ensuing osmotic shrinkage, which is only effective after the lumen has lost its connection to the extracellular medium, and from the voltage and pH dependence of ASOR that shuts it down at the plasma membrane. Likewise, ablation of ASOR/TMEM206, which is also found on phagosomes (Extended Data Fig. 6b), did not impair the uptake of fluorescent beads by phagocytosis (Extended Data Fig. 6c).

We examined the localization of another candidate, heteromeric LRRC8/VRAC (ref. 11) (Fig. 1e,g). Both LRRC8A and LRRC8D subunits localized to the plasma membrane and variably to cytoplasmic puncta in BMDMs from mice expressing epitope-tagged subunits (Extended Data Figs. 7a,b and 8a,b) and in transfected HeLa cells (Extended Data Fig. 8d). Contrasting
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Low Cl–

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With a recent report\(^1\), these puncta did not co-localize with lysosomes. Whereas LRRCK8D was not found on MPs (Extended Data Fig. 8c), LRRCK8A was detected on MPs in less than 10% of the cells 4 min, but not 7 min, after M-CSF stimulation (Extended Data Fig. 7c). Disrupting the essential LRRCK8A subunit\(^{11,12}\) had no significant effect on MP resolution (Extended Data Fig. 5e). Small effects, however, might have been missed as Lrrc8a expression was reduced by only ~60% in Cx3cr1-Cre\(^{12}\); Lrrc8a\(^{lox}\)lox macrophages (Extended Data Fig. 5f).

We asked whether other Cl– conductors might replace ASO\(^{2}\) in MP resolution. Overexpression of CIC-2, whose disruption did not affect MP resolution (Extended Data Fig. 5a), failed to rescue resolution (Fig. 4i). This failure might be due to its voltage dependence\(^{3}\), which is opposite to that of ASO\(^{2}\) and predicts low currents in lumen-negative MPs. However, transfection of the aldosteronism-causing CIC-2(G24D) mutant\(^{4}\), which yields large linear Cl– currents, had no effect either (Fig. 4i). CIC-2, a plasma membrane channel, may have been excluded from MPs. We therefore

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**Fig. 4 | MP resolution impaired by Tmem206 ablation and rescued by Cl– channels.** a–c, Tmem206 disruption in BMDMs impairs MP resolution, \(n = 7\), \(N = 419\) (WT); \(n = 11\), \(N = 922\) (Tmem206\(^{−/−}\)). Impact of Cl– substitution on WT resolution (green dashed line, data from Fig. 1c) shown for comparison. Data included in WT control partially overlap with NaCl control in Fig. 1c. Two-tailed Mann–Whitney test (volume), unpaired two-tailed \(t\)-test (intensity).

d–f, KD of Tmem206 in HT-1080 cancer cells impairs MP resolution, \(n = 5\), \(N = 37\) (control, transfected with non-targeting siRNA), \(N = 38\) (Tmem206\(^{−/−}\)). One-sample two-tailed \(t\)-test comparing ratio (KD and corresponding control) with hypothetical value of 1. g, Tmem206, but not dead-pore mutant Tmem206(K319C) (ref. \(^{1}\)), rescues MP resolution of Tmem206\(^{−/−}\) BMDMs. \(n = 5\), \(N = 37\) (WT + GFP); \(n = 5\), \(N = 10\) (WT + Tmem206); \(n = 4\) (Tmem206\(^{−/−}\) + GFP \(N = 11\) or + Tmem206 N = 9); \(n = 5\), \(N = 16\) (Tmem206\(^{−/−}\) + Tmem206(K319C)); Kruskal–Wallis comparison with Dunn’s post-hoc test with regard to Tmem206\(^{−/−}\) + GFP done on cells for every minute, significance shown between Tmem206\(^{−/−}\) + GFP (\(N = 8\)) and Tmem206\(^{−/−}\) + Tmem206(K319C) (\(N = 10\)) is not significantly different from Tmem206\(^{−/−}\) + GFP. h, Overexpression of WT and Y10A mutant Tmem206 in Tmem206\(^{−/−}\) macrophages similarly enhances MP shrinkage, \(n = 4\), \(N = 98\) (NT), \(N = 31\) (Tmem206\(^{−/−}\) + Tmem206), \(N = 28\) (Tmem206\(^{−/−}\) + Tmem206(Y10A)). i, Neither CIC-2 nor ‘open’ CIC-2(G24D) (ref. \(^{24}\)) rescues resolution in Tmem206\(^{−/−}\) BMDMs. \(n = 4\), \(N = 43\) (NT); \(n = 4\) (Tmem206\(^{−/−}\) + CIC-2 N = 13 or + CIC-2(G24D) N = 12). j, CIC-5(E211A) uncoupled mutant\(^{28}\), but not WT CIC-5, rescues Tmem206\(^{−/−}\) MP resolution. \(n = 3\), \(N = 8\) Tmem206\(^{−/−}\) + CIC-5, \(n = 4\), \(N = 9\) + CIC-5(E211A), \(n = 4\), \(N = 40\) NT; one-way ANOVA with Dunnett’s post-hoc test (with regard to NT) done on cells for every minute. Significance shown between Tmem206\(^{−/−}\) (NT) (\(N^* = 20\)) and Tmem206\(^{−/−}\) + CIC-5(E211A) (\(N^* = 8\)). \(P = \leq 0.05\), \(\ddagger = \leq 0.01\), \(\ddagger\ddagger = \leq 0.001\) and \(\ddagger\ddagger\ddagger = \leq 0.0001\). All plots present mean ± s.e.m. (shown as bands). \(N^*\) number of cells. Source numerical data are available in source data.
used the E211A CIC-5m-uncoupled, channel-like mutant of the early endosomal anion–proton exchanger CIC-5 (ref. 25), which mediates a pure, voltage-independent Cl− conductance26–28. Unlike WT CIC-5, CIC-5m-unc rescued resolution of Timem206+/− MPs (Fig. 4j). Hence, ASOR can be replaced by another appropriately targeted Cl− conductance, but not by a 2Cl−/H+ exchanger.

Role of luminal acidification. ASOR/TMEM206 needs both cytoplasmic positive voltages and extracellular (or luminal) acidity25,28 for activity. In the absence of other conductances, macropinosomal Na+ channels together with an inside-out tenfold Na+ gradient would drive the luminal potential (referred to cytoplasm) to ~60 mV, a value amply sufficient for ASOR activity25,26. Agreeing with previous work29, 10 min after M-CSF stimulation, luminal pH reached ~5.5 (Fig. 5a). This value is close to pHo of ASOR (~5.3) (refs. 15,16). If ASOR retains its pH dependence in MPs, luminal alkalinization should inhibit MP shrinkage. Indeed, extracellular NH4Cl, which alkalinizes cellular compartments, abolished vesicle resolution (Fig. 5b,c).

V-type H+–ATPases acidify diverse intracellular organelles30. In macrophages, which express proton pumps at the plasma membrane31, they might reach MPs directly from the outer membrane and/or by vesicular fusion. The lysosomal a3 proton pump subunit32, against which good antibodies are available33, reached MPs when they acquired CIC-7 and rab7 (Extended Data Fig. 9). Proton pump inhibition with bafilomycin A partially alkalinized MPs already at the beginning of the measurement (Fig. 5d) and partially inhibited MP resolution (Fig. 5f,g).

Both luminal Cl− removal and disruption of ASOR/TMEM206 led to a marked luminal acidification (Fig. 5a). Both procedures should increase the driving force for electrogenic H+ uptake by making the lumen more negative: in WT, low luminal Cl− led to a marked luminal acidification (Fig. 5a). Both procedures Cl− independent of proton pump activity (Fig. 5e). Whereas luminal Cl− removal and disruption of ASOR/TMEM206 led to a marked luminal acidification (Fig. 5a), both procedures should increase the driving force for electrogenic H+ uptake by making the lumen more negative: in WT, low luminal Cl− caused ASOR-mediated influx of negatively charged Cl−, whereas ASOR knockout (KO) prevents the efflux of Cl− driven by lumen-to-cytosol Cl− gradients. The predicted negative shift in luminal potential might enhance H+–ATPase activity. However, since ASOR KO induced acidification also in the presence of bafilomycin (Fig. 5d), there must be additional electrogenic acidification mechanisms.

Attractive candidates are CLC 2Cl−/H+ exchangers that couple vesicular H+ uptake to Cl− efflux. The lumen-negative voltage predicted for Timem206+/− MPs would not only increase the electrochemical potential for H+ entry and Cl− efflux, but also activate CLC exchangers that open at cytoplasmic potentials > +20 mV (ref. 3). To test for CLC anion/proton exchange, we imposed opposite Cl− gradients on Timem206+/− MPs and measured luminal pH. Assuming that without ASOR most, if not all, Cl− crosses the membrane through CLC 2Cl−/H+ exchangers, we predicted that lumen-to-cytosol and cytosol-to-lumen Cl− gradients will accumulate or deplete, respectively, luminal H+. Indeed, luminal pH was more acidic with high than with low luminal Cl− concentration, and this effect was independent of proton pump activity (Fig. 5e). Whereas luminal Cl− removal alkalinized Timem206+/− MPs, it acidified WT MPs (Fig. 5a). This surprising contrast likely results from a large difference in MP potential, generated by the ASOR Cl− conductance, between high and low luminal Cl− conditions (Fig. 5i and Supplementary Note Fig. 3a,b). The resulting lumen-negative voltage in the presence of ASOR provides a favourable driving force for electrogenic H+ influx with low luminal Cl−.

Having demonstrated macropinosomal chloride/proton exchange activity (Fig. 5c), we sought the underlying CLC exchanger. We had already excluded late endosomal/lysosomal CIC-7 (Figs. 2 and 3 and Extended Data Fig. 5d). The predominantly neuronal CIC-6 (ref. 28) was almost absent from BMDMs (Fig. 11). CIC-3 and CIC-4 were barely detectable in BMDMs (Fig. 11j), reside in late endosomes24,28 and did not affect MP resolution (Extended Data Fig. 5).

By contrast, CIC-5 is robustly expressed in macrophages (Fig. 1k) where it co-localized with early endosomal markers (Fig. 6b) as in other cells24,27. Moreover, the rescue of Timem206+/− MP resolution by CIC-5m-unc (Fig. 4j) suggested that CIC-5 reaches MPs at least when overexpressed. Upon M-CSF stimulation, native CIC-5 was indeed detected on large MPs, where it co-localized with ASOR/TMEM206 (Fig. 5j,k), rab5 and later rab7 (Fig. 6c,d). CIC-5 might facilitate MP resolution directly by mediating Cl− efflux and indirectly by activating ASOR through Cl−-gradient-driven acidification. However, Clcn5 disruption failed to significantly decrease MP resolution even when H+–ATPases were inhibited (Fig. 5g and Extended Data Fig. 5c) and no effect of Clcn5 disruption on luminal pH was observed (Fig. 5h and Extended Data Fig. 10c), suggesting further redundancies in acidification mechanisms. Theoretically, even an H+ conductance may acidify vacuoles by up to one pH unit when they are depolarized by TPCs and the tenfold lumen-to-cytosol Na+ gradient. However, a sizeable parallel Cl− conductance (embodied by ASOR) would render the lumen more positive and reduce the acidification achievable by an H+ conductance.

Model for MP resolution. Our results suggest a model for MP resolution (Fig. 7a,b) that incorporates as main players previously implicated TPC channels3, newly identified ASOR/TMEM206, CIC-5 and H+–ATPases, in addition to other H+ transporters such as an H+ conductance and a high water permeability. Both CIC-5 and ASOR are strongly outwardly rectifying and thus virtually inactive at physiological plasma membrane voltages. ASOR shows higher plasma membrane expression than CIC-5 (ref. 3) and might be directly incorporated into MPs upon their formation even when lacking an N-terminal endosomal targeting motif3. Both CIC-5 and ASOR probably reach MPs also by fusion with endosomes. MP resolution is triggered by Na+ efflux through newly inserted endosomal TPCs that changes the voltage from lumen positive to lumen negative. This depolarization directly activates CIC-5 and potentially other CLCs. It is also permissive for ASOR activity, which, however, additionally needs luminal acidification. The lumen-negative voltage also increases the electrochemical driving force for CLC-mediated Cl− efflux and H+ uptake. Together with TPC-mediated Na+ efflux, CLC-mediated efflux of Cl− probably contributes to MP shrinkage, aided by the fact that counter-transported H+ is osmotically inactive as it binds to buffers. A direct contribution of CIC-5 to resolution, however, is much smaller than that of ASOR, as evident from our failure to observe an effect of Clcn5 disruption on MP resolution (Extended Data Fig. 5c). A more important effect of CIC-5 may be a contribution to luminal acidification, which in turn activates ASOR. Na+ efflux through TPCs, and electrically coupled Cl− efflux through ASOR (with a minor contribution of CLCs), causes luminal loss of Na+ and Cl− and osmotic shrinkage. This shrinkage, in turn, prevents a rapid decline of luminal Na+ and Cl− concentrations, maintaining appropriate gradients for further shrinkage, which gradually decrease with increasing luminal concentrations of non-transported osmotics.

The feasibility of this transport scheme was tested in a reductionist mathematical model (Supplementary Note and Supplementary Note Figs. 1–4). It considers the voltage and pH dependencies of ASOR and CLCs (Supplementary Note Fig. 2), assumes infinite water permeability and neglects insertion or removal of transporters over time. The model semi-quantitatively predicts MP shrinkage, suggests a (rather minor) role of CLC exchangers during early phases when the less acidic luminal pH favours transport through CLCs rather than ASOR (Supplementary Note Fig. 3a). Later CLCs will operate close to electrochemical equilibrium and are partially inhibited by luminal acidification. The model explains the opposite effects of luminal Cl− removal on luminal pH in the presence (Fig. 5i and Supplementary Note Figs. 1b,d and 3a) and absence of ASOR (Fig. 5i and Supplementary Note Figs. 1c and 3b–d). This difference mainly results from changes in membrane potential.
Fig. 5 | Roles of V-type ATPase and CLC 2Cl−/H+ exchangers in luminal pH and resolution of MP. a, Disruption of Tmem206 or low luminal Cl− acidify MP lumina. n = 3, N = 81 (WT); n = 7, N = 142 (Tmem206−/−); n = 4, N = 150 (low Cl−); n = 5, N = 198 (low Na+). b, c, Addition of 20 mM NH4Cl abolishes MP shrinkage, n = 4 (N = 419 WT, N = 749 WT + NH4Cl). d, Baflomycin (BafA) alkalinizes MPs in both WT and Tmem206−/− BMDMs. n = 4 (WT alone N = 175 or +BafA N = 188); n = 3, N = 117 (Tmem206−/−); n = 4, N = 194 (Tmem206−/− + BafA). e, pH changes upon lowering luminal Cl− from 159 mM to 9 mM in Tmem206−/− BMDMs indicate Cl−/H+ exchange (n = 3; high Cl− alone N = 287, +BafA N = 429, low Cl− alone N = 298, +BafA N = 369). f, g, BafA decreases MP shrinkage in WT and Clcn5−/−, but not Tmem206−/− BMDMs n = 7, N = 456 (WT); n = 8, N = 468 (WT + BafA); n = 3 (Tmem206−/− alone N = 359 or +BafA N = 278); n = 5 (Clcn5−/− alone N = 421 or +BafA N = 434). h, Baflomycin alkalinizes WT and Clcn5−/− MPs. n = 4 (WT alone N = 480 or +BafA N = 384); n = 6 (Clcn5−/− alone N = 433 or +BafA N = 480). i, Reductionist mathematical model (Supplementary Note) semi-quantitatively explains results shown in a and e. Overlay from pH panels of Supplementary Note Fig. 3a,b. Shaded area, early timepoints that could not be determined experimentally. j, k, Tmem206 and Clcn5 are co-expressed on same MPs, at both 4 and 7 min after M-CSF. Small panels, magnified MPs indicated by arrowheads. Pearson’s R calculated from 25 cells (4 min), 16 cells (7 min) from three (4 min) and two (7 min) independent experiments. All plots present mean ± s.e.m. (shown as bands) averaging from individual mice. Scale bars, 10 μm and 1 μm for the magnification panels. In a, d, e and h, 20 mM NH4Cl was added to show dye responsiveness to alkalinization (bleaching control). Source numerical data are available in source data.

The model also shows that CLCs, without ASOR but together with TPCs, can in principle support vesicle shrinkage with or without additional acidifying transporters (Supplementary Note Fig. 3d), albeit less efficiently than ASOR (Supplementary Note Fig. 3a). It also predicts that resolution is largely independent of the particular combination of acidifying transporters (CLCs, H+−ATPase and H+ conductance) (Supplementary Note Fig. 3a, e-j). Even a model vesicle containing only an H+ conductance together with TPC and ASOR showed marked shrinkage, but at a slightly lower rate owing to reduced luminal acidification (Supplementary Note Fig. 3j). A hint for such a conductance comes from only partially impaired MP shrinkage in baflomycin-treated Clcn5−/− BMDMs (Fig. 5f,g) and the acidification upon luminal Cl− removal (Fig. 5a), which cannot be caused by anion/proton exchangers (Supplementary Note Fig. 3h). However, it can be explained by V-type-ATPases or an H+ conductance (Supplementary Note Fig. 3j) that respond with increased H+ transport to more negative luminal potentials. The H+ conductance might be embodied by the Hv1 H+−channel18,39, which, reminiscent of ASOR and Clcn5, is strongly outwardly rectifying.
The relative insensitivity of volume changes to acidification mechanisms may result from feedback loops that control ASOR and CLC activity. ASOR Cl− currents oppose the depolarizing, TPC-mediated Na+ currents, resulting in a hyperpolarization that directly inhibits ASOR in a negative feedback. This hyperpolarization also reduces the electrochemical driving force for H+ entry and inhibits the activity of CLC exchangers, which show a voltage dependence similar to ASOR. Both effects result in

Fig. 6 | CIC-5 co-localizes with TMEM206 in macrophages endosomes and MPs. a, CIC-5 immunostaining in WT and Clcn5−/− (control) BMDMs. b, CIC-5 was co-localized with endolysosomal markers rab5 and EEA1, partially with late endosomal marker rab7 but not with lysosomal LAMP1. c, d, CIC-5 is present on MPs labelled by rab5 and rab7 stainings, at 4 min (c) and 7 min (d) after M-CSF addition. Pearson’s R calculated from 31 cells (Rab5, 4 min), 17 cells (Rab5, 7 min), 21 cells (Rab7, 4 min) and 15 cells (Rab7, 7 min). e, TMEM206 (green) and CIC-5 (red) are present on the same vesicles in BMDMs. Absence of CIC-5 antibody labelling in Clcn5−/− BMDMs confirms the specificity of staining. Pearson’s R calculated from 29 cells from two (Rab5 and CIC-5/TMEM206) and one (Rab7) independent experiments. Mean ± s.e.m. Scale bars, 10 µm and 1 µm for the magnification panels. Source numerical data are available in source data.

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Fig. 7 | Physiological role of ASOR. a, Model for roles of TPC, CIC-5, ASOR and V-ATPase in MP shrinkage. The voltage $U$ is defined as difference between the luminal and cytoplasmic electrical potentials ($U_{\text{lum}}$ and $U_{\text{cyt}}$, respectively). b, Effects of anion transporter disruption on MP shrinkage. $n=19$ (WT); $n=11$ (Tmem206–/–); $n=3$ (Clcn2–/–, Clcn3–/– and Clcn4–/–); $n=5$ (Clcn5–/–); $n=3$ (Clcn7–/–); $n=4$ (Lrcc8a–/–); $n=7$ (WT + DMSO); $n=8$ (WT + BafA). Kruskal–Wallis (Dunn’s post hoc) test for all KO versus WT (includes WT controls to all corresponding KO in the same dataset) with Kruskal–Wallis not only consistent with our model, but also excludes that the failure of both voltage- and pH-mediated negative feedback loops predicts that ASOR currents, and hence MP resolution, should be resilient to ASOR expression levels beyond a certain threshold (Supplementary Note Fig. 4).

Agreeing with this prediction, ASOR overexpression in WT or Tmem206–/– macrophages failed to significantly enhance MP resolution beyond WT rates (Fig. 4g). The model predicts that an ASOR mutant with alkaline-shifted pH dependence leads to faster MP resolution by loosening the ‘pH brake’ on ASOR activity (Supplementary Note Fig. 4a). We resorted to the R87C mutant that yields notable currents already at pH 7.4 and displays WT-like rectification18. Transferring this mutant into Tmem206–/– cells (to avoid mutant/WT heteromers of the trimeric channel18,19) indeed accelerated vesicle resolution compared with WT (Fig. 4c). This is not only consistent with our model, but also excludes that the failure of WT ASOR overexpression to accelerate resolution is due to a rate-limiting magnitude of Na+ (TPC) currents.

Importantly, if both ASOR and CLCs lacked their steep voltage and pH dependencies, they might dominate over the TPC-mediated Na+ conductance and clamp luminal pH to near-neutral or even alkaline values (Supplementary Note Fig. 1a, b), which may interfere with many processes that depend on acidic luminal pH. Collectively, our work suggests an intricate coupling between TPCs, ASOR and CLCs that involves several feedback loops that are enabled by the strong modulation of ASOR and CLCs by voltage and pH, conserved properties whose ‘purpose’ has remained enigmatic.

Osmotic shrinkage of MPs facilitates budding of vesicles through the formation of tubular extensions that bind BAR-domain proteins4,42. These vesicles are needed to recycle plasma membrane proteins back to the cell surface. Recycling is especially important for macropinocytosis because it internalizes large chunks of plasma membrane. Hence, impaired MP shrinkage may lower the abundance of surface receptors4. Hypothesizing that this includes the receptor for the C5a complement, which stimulates macrophage migration4, we compared migration of WT and Tmem260–/– BMDMs in the absence and presence of C5a. Consistent with our hypothesis, migration of Tmem260–/– BMDMs was mildly reduced compared with WT in the presence, but not in the absence, of C5a (Fig. 7d).

Macropinocytosis of extracellular proteins is used by many cancer cells to supply amino acids under nutrient starvation4,43,44. This process has often been studied in MIA PaCa-2 human pancreatic cancer cells44, but operates also in other tumours displaying RAS mutations4,45 that stimulate constitutive macropinocytosis4.

luminal alkalization that additionally inhibits the strongly pH-dependent ASOR (Supplementary Note Fig. 4). The combination of both voltage- and pH-mediated negative feedback loops predicts that ASOR currents, and hence MP resolution, should be resilient to ASOR expression levels beyond a certain threshold (Supplementary Note Fig. 4).

Significance shown for 14 (Tmem260–/– + Tmem206(R87C)) and 9 (WT NT) cells from n = 3 mice each ($N =$ 22 WT NT, $N =$ 26 Tmem206–/– NT, $N =$ 17 Tmem206–/– + Tmem206(R87C) MPs). * $P \leq 0.05$, ** $P \leq 0.01$. d, Tmem206–/– macrophages migrate slower than WT in a scratch assay in presence of C5a. Data obtained 12 h after scratch. $n=13$, one sample two-tailed t-test comparing ratio (WT to corresponding KO) with hypothetical value of 1. Lines connect values obtained from WT and KO BMDMs that were prepared, handled and imaged in parallel. e, Tmem206 disruption enhances the positive effect of 3% BSA on viability of MIA PaCa-2 cancer cells in amino-acid-depleted medium measured after 72 h. $n=3$, cells generated with two sgRNAs; two independent cell lines (open and filled symbols) for sgRNA2, unpaired two-tailed t-test. For all plots, mean ± s.e.m. is shown. Source numerical data are available in source data.
Using clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 genomic editing, we generated several independent TMEM206 KO mice and control edited TMEM206+/+ MIA PaCa-2 lines and assayed their survival in amino-acid-depleted medium with or without 3% bovine serum albumin (BSA) (Fig. 7e). In all cases, addition of BSA increased the number of TMEM206 KO cells relative to TMEM206+/+ control cells. These results are consistent with unchanged rate of MP formation in TMEM206 KO cells (Extended Data Fig. 6a) in conjunction with impaired recycling of internalized albumin and increased MP acidification (Fig. 5a,i). The slow resolution of KO MPs may give them more time to fuse with degradative lysosomes while still containing substantial amounts of albumin and being over-acidified, leading to an increased cellular supply of amino acids. Decreased ASOR activity might hence contribute to the growth of certain cancers. Indeed, The Cancer Genome Atlas database (https://www.proteinatlas.org/) suggests that low TMEM206 expression weakly correlates with decreased survival of patients with pancreatic cancer that displays a high prevalence of KRAS mutations18.

**Discussion**

Previous studies focused on plasma-membrane-resident ASOR and its detrimental role in acid-induced cell death and stroke19,20,21. It now emerges that its main physiological function is in the endocytic pathway. In addition to MPs, ASOR/TMEM206 is found on endosomes16. Similar to our results for MPs, endosomes were more acidic in Tmem206+/− HEK cells7. Transferrin endocytosis was increased in Tmem206−/− cells owing to increased transferrin receptor recycling1, arguing against the hypothesis that ASOR-mediated endosome shrinkage increases endocytic trafficking. An important difference between MPs and endosomes is their initial luminal ion composition. Endosomal Cl− concentration is initially low (~15 mM) in several cell types and progressively increases over time in parallel to acidification14,15. This increase depends on CIC-5 (ref. 22) and CIC-3 (ref. 19) for early and late endosomes, respectively. The low initial Cl− concentration was attributed to negative surface charges that can decrease luminal anion concentrations in small endosomes, but not in vastly larger MPs. Unlike MPs, endosomes may accumulate Cl− into their lumen through both CLCs23,24 and ASOR in a process driven by proton pumps. Model calculations15 and analysis of mice expressing mutant CLCs mutated into uncoupled Cl− conductors26,35 show that both Cl− channels and 2Cl−/H+ exchangers support proton-pump-driven acidification and luminal Cl− accumulation, with exchangers being more efficient than channels. The described effect13 of ASOR KO on endosomal pH and Cl− may reflect the higher efficiency of CLCs versus Cl− channels in active acidification36 rather than luminal Cl− loss. Once endosomes have acquired sufficiently high luminal Cl− concentration, they might use ASOR and TPCs for shrinkage and vesicle budding as found here with MPs.

In conclusion, acid-activated ASOR/TMEM206 Cl− channels, together with luminal acidification and previously identified TPCs, are crucial for MP shrinkage (Fig. 7a). MPs express additional, minor Cl− exit pathways such as CIC-5 (Fig. 5j,k and Supplementary Note Fig. 3d) because they shrink, albeit at a much slower rate, also in the absence of ASOR. ASOR operates in vesicle-intrinsic feedback loops that render MP pH and resolution remarkably resilient towards variations in transporter copy numbers during membrane remodelling by vesicle fusion and budding, processes that are difficult to balance with local variations of vesicle densities. Our work provides a comprehensive framework for understanding an important aspect of macropinocytosis, an uptake mechanism that is not only crucial for immune cells, but also for nutrient uptake in cancer cells14,15. Ion concentration- and voltage-based feedback loops as those described here may play roles in other vesicular compartments.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41556-022-00912-0.

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Methods
All animal experiments, including the generation of new mouse lines, were approved by Berlin authorities (LAGeSo). Animals were housed under standard conditions (12 h/12 h dark-light cycle with food and water ad libitum) in the MDC animal facility according to institutional guidelines. In all experiments, 8- to 22-week-old (8-16 weeks old for BMDM preparation) C57BL/6 mice (Mus musculus) of both sexes were used.

Generation of Tmem206 KO mice. Tmem206−/− mice were generated using CRISPR-Cas9 genome-editing technique. Different combinations of guide RNAs (g1, g2, g5 or g6) were injected by pronuclear microinjection in fertilized C57BL/6J mouse oocytes that were implanted into foster mothers by the MDC Transgenic Core Facility. g1 (GCTGAGGAGAAGCGAGGAC) and g5 (CAAGCTCCAGGTTGTGCCG), both targeting exon 2 that codes for the Tmem206 N terminus, were injected together, which led to a frame-shifting 62 bp deletion and a subsequent smaller fragment of 27 bp. Similarly, either g1 or g2 (GCTGAGGAGAAGCGAGGAC) and target exon 2 were injected together with g6 (CCGGATGCTGGTGTAGAC) targeting exon 3, which in both cases led to slightly different deletions of ~10 kb. These injections led to the generation of three different Tmem206 KO mouse lines. Absence of protein expression was validated by western blot using custom-made anti-Tmem206 antibodies.

Generation of Tmem206 antibodies. Polyclonal antibodies against Tmem206 were raised in rabbits against the peptide VKTKEEDGREAVEFRQET (Merck, 11836145001) and the following antibodies: rat anti-LAMP1 (BD Pharmingen, 553792; 1/500), sheep anti-EEA1 (R&D Systems, AF8047; 1/200) and mouse anti-Rab9A (BioLegend, 376529; 1/500) were used to stain BMDMs from two to three animals. On average, 15 cells per condition were analysed.

By to study VRAC localization in HeLa cells, these were transfected with untagged Lrrc8a and TdTomato-tagged LRRC9D (1:1) and DNA1.1 backbone vector using Lipofectamine 2000 (Invitrogen, 11668-019). One day after transfection, cells were fixed with PFA 4% and LRRC9A/LRRC9D heteromers were detected using rabbit anti-RFP antibody (Rockland, 600-401-379; 1/500). Samples (30 μg protein) were separated by SDS–PAGE and blotted. Detection used our anti-Tmem206 antibody (1/1,000, against extracellular loop), rabbit anti-CIC-2 (ref. 1) (1/1,000), rabbit anti-CIC-3 (ref. 2) (1/500), rabbit anti-CIC-4 (ref. 3) (1/200), rabbit anti-CIC-5 (ref. 4) (1/200), rabbit anti-CIC-6 (ref. 5) (1/500), rabbit anti-CIC-7 (ref. 6) (1/100) and rabbit anti-LRRC9A (ref. 7) (1/1,000). Na/K ATPase α1 subunit and actin (loading controls) were detected with a mouse anti-Na/K ATPase α1 subunit antibody (Millipore, #36-05; 1/10,000), and mouse or rabbit anti-actin antibody (Sigma, A2282 and A2666; 1/1,000 for both), respectively.

Macropinocytosis live imaging. Twenty-four hours before imaging, 1.5×10^6 BMDMs were seeded in M-CSF-free complete DMEM on the glass plate of uncoated glass-bottom 35 mm dishes (MatTek, P35G-1.5-10). First, medium was replaced by a live cell imaging buffer containing (in mM): 150 NaCl, 1 MgCl₂, 1 CaCl₂, 5 glucose and 20 HEPES. pH 7.2 and NaCl concentration was kept constant (200 mM) while the concentration of MgCl₂ was adjusted to 1-3 mM. Currents were sampled at 1 kHz and low-pass filtered at 10 kHz. The pipette solution contained (in mM): 150 CsCl, 10 EGTA, 10 HEPES and 5 MgCl₂, pH 7.2 with CsOH (320 mM Osom 3000). The pH 7.4 bath solution contained (in mM): 145 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES pH 7.4 with NaCl (320 mM osmol/kg), adjusted with mannitol. Solution at pH 4.8 was buffered with 5 mM citric acid and 50 mM NaOH. From pH 7.2 to 4.8, the holding potential of −30 mV, the voltage was clamped in 1-s-long 20 mV steps from −80 mV to +80 mV, preceded and followed by a 0.5 s step to −80 mV every 4 s.

Immunocytochemistry. WT and Tmem206−/− HEK923 cells were seeded on glass coverslips and fixed after 1 day with 4% paraformaldehyde (PFA). After antigen retrieval (citrate buffer, pH 6 at 95 °C for 10 min), anti-CIC-5 (rabbit, 1/250, ref. 8) or guinea pig (1/100, ref. 9) and against the a3 subunit of the H+-ATPase (guinea pig, 1/100, ref. 10). For CIC-3, LRRC9A and LRRC9D, we used different knock-in mice previously generated in our group: Venus-tagged CIC-3 (ref. 11), SV40-HA-tagged LRRC9A (ref. 12) and TdTomato-tagged LRRC9D (ref. 13). Proteins were detected using chicken anti-βGEP (Aveslab, GFP-1020; 1/500), rabbit anti-HA (Cell Signaling, 3724; 1/500) and rabbit anti-RFP (Rockland, 600-401-379; 1/500) antibodies, respectively. For immunohistochemistry of newly generated MPs, BMDMs were cultured in M-CSF (10 ng/ml) for 4 days and these cells were then fixed (time point 4 min), or fixed 3 min before replacing the M-CSF containing medium by complete medium (without M-CSF) (time point 7 min). Key stainings were done with BMDMs derived from two to three animals. On average, 15 cells per condition were analysed.

To study VRAC localization in HeLa cells, these were transfected with untagged Lrrc8a and TdTomato-tagged LRRC9D (1:1) in pDNA1.1 backbone vector using Lipofectamine 2000 (Invitrogen, 11668-019). One day after transfection, cells were fixed with PFA 4% and LRRC9A/LRRC9D heteromers were detected using rabbit anti-RFP antibody (Rockland, 600-401-379; 1/500).
30 s for overexpression experiments). In pl measurements with 70 kDa Oregon Green 488–ex20, 20 mM NH4Cl was added after 15 min to alkalize the MP as a control for Oregon Green bleaching. Where stated, V-ATPas was inhibited with 100 nM bafilomycin A1 (Alfa, A851585) (10 min pre-incubation before M-CSF stimulation, and throughout the rest of the experiment). Where stated, MPs were alkalized with 20 mM NH4Cl added together with M-CSF. After washing, 20 mM NH4Cl remained in the imaging buffer.

Imaging was performed with a Nikon spinning disc microscope (Yokogawa spinning disc) combined with a CFI-UPL objective lens (Nikon) with a live cell imaging buffer of 37°C (Okolab incubator) every minute in 2 stacks with 0.6 µm steps on 60X oil objective (Plan-Apo, NA 1.40, Nikon) with an additional 2X magnification on an EMCCD Camera (AU-888, Andor). Depending on fluorescence, the following lasers were used: excitation 488 nm (emission 525/30 nm) for GFP, excitation 515 nm (emission 540/35 nm) for YFP, excitation 561 nm (emission 600/50 nm) for TMEM206. Live cell images were recorded with 200 ms exposure. Where Oregon Green 488, two excitation wavelengths were used: 445 nm and 515 nm with emission at 540/30 nm. Final pixel size was 0.11 µm.

For MP shrinkage in HT–1080 cells, these were transfected with ON-TARGETplus human siRNA SMART pool against TMEM206 (55248) (Dharmacon, L-010876-02-0005) or non-targeting control pool (Dharmacon, D-008180-10-05) with Lipofectamine 3000 transfection reagent (Invitrogen, L3000105) according to the manufacturer protocol. Forty-eight hours post-transfection, cells were seeded in complete DMEM on uncoated glass-bottom 35 mm dishes (MatTek) and imaged 24 h later. Sixteen hours before imaging, cells were transferred to DMEM. Human EGF (PeproTech, AF-100-15; 200 ng ml−1) was used to stimulate micropinocytosis. Imaging and tracking of MPs was done as for MDMs. After each experiment, knocking down (KD) efficiency was verified by western blot.

Oregon Green calibration. BMDMs were seeded as for resolution or pH experiments. Cells were stimulated with imaging buffer containing 10 ng ml−1 recombinant murine CSa (PeproTech, 315–40) or in a CsA-free DMEM. The cell monolayer was scratched with a 10 µl pipette tip and immediately placed on the microscope for imaging. Phase contrast imaging of the scratch and surrounding cells was performed on a Nikon Ti Eclipse microscope operated with NIS software in a Tวน 35 mm dish (MatTek) for 2 h on 20x air objective (Plan Apo NA 0.75, Nikon) with back-illuminated SCMOS camera (Prime95B, Photometrics) using Nikon Volume Contrast function. Final pixel size was 0.55 µm.

Data analysis of macropinocytosis experiments. We began by reducing every three-dimensional stack of images to a two-dimensional maximum projection along the z axis (using ImageJ’s native maximum projection function). Further analysis used Python (version 3.8) scripts. Each image sequence was analysed starting from the last timepoint, tracking MPs with high signal through subsequent z-projected images back to the first time step where signal-to-noise ratios and detection efficiency were much lower.

For the detection of MPs, intensity histograms of each image were truncated at the upper 95th and bottom 15th percentiles to distinguish MPs from background and remove outlier pixels. The histogram-truncated image was then segmented using an inverse_gaussian_gradient filter (scikit-image function skimage.feature.inverse_gaussian_gradient, parameters alpha = 100, sigma = 5 pixels), followed by thresholding the image below the 20th percentile of the intensity distribution (of the inverse filtered images). Then images were thresholded using skimage.filters.threshold_local function of scikit-image (parameters: block size 41, offset 10). Further, we performed morphological opening of the image (erosion followed by a dilation) using skimage morphology:opening function with disk footprint with 40 px radius following by two morphological reconstructions of the image (first dilation, then erosion, skimage.morphology:reconstruction function) to separate scratch area from surrounding cells. We detected the borders of the scratch on this image mask and used the findContours and drawContours functions in the OpenCV package (version 4.2.0) and selecting the longest detected contour. Using the same mask for the original scratch area, we detected and counted the number of cells at every following time step image by filtering the image with a sequence of difference of Gaussian (DoG) filters (scikit-image package, DoG function skimage.feature.blob dog) with minimum and maximum standard deviations of the Gaussian kernels used set to 5 and 30 pixels correspondingly and absolute lower bound for scale space maxima set to 0.02. Finally, we subtracted the number of cells detected on the scratch area at t = 0 from number of cells detected at every following timeframe and normalize this number to the original area of scratch (calculated in number of pixels and further converted to µm2).

MIA PaCa-2 2 TMEM206 KO generation. Clonal MIA PaCa-2 KO cell lines were generated using CRISPR-Cas9 with two different sgRNAs: sgRNA1 (GAACCGAGAAGAGCTCTTTC) and sgRNA2 (CAGCGTGAACAGCATTACG) as described previously for HEK cells.21 KO was verified by Sanger sequencing and western blot. For growth assays, six clones were picked: three KO clones (SB-1G11 Δ# nucleotides, F5-1A2 Δ1 and +1 nucleotides, and F5-1A7+1 nucleotide) and three clones that went through the transfection process but remained WT.

MIA PaCa-2 growth assay. Twenty-four hours before experiments, cells were seeded on 96-well plate in quadruplicates at 30% density in complete DMEM. The next day, the medium was replaced with one of following: 1) amino-acid-free DMEM (Genaxxon, C4150.0500) supplemented with 5% essential amino acids (Merck, 55540), 10% dialysed FBS (Pan Biotech, P30-2101), 1% penicillin–streptomycin and additional 1 mM CaCl2, (final Ca2+ concentration 2 mM) or 2) amino-acid-free DMEM supplemented with 5% essential amino acids, 10% dialysed FBS, 1% penicillin–streptomycin, 3% BSA (Pan Biotech, P60-10200) and additional 3 mM CaCl2 (final Ca2+ concentration 4 mM). Ca2+ concentration was adjusted in B2 medium solutions to stimulator free Ca2+ solution (known to markedly bind Ca2+ (ref. 22)) because constitutive macropinocytosis is stimulated by extracellular free Ca2+ through Ca2+-sensing receptors.23 After 72 h incubation at 37°C and 5% CO2, we evaluated cell survival in both conditions with XTT assay (Cell Signaling, 9095) measuring absorbance at 450 nm with an absorbance reader (ASYS Hitachi) as readout. Data are presented (Fig. 7) as absorbance ratios between BSA-exposed and unexposed cells.

Phagocytosis of beads. WT and Tmem206−/− BMDMs were seeded on coverslips for assessing phagocytosis in situ. The day after, yellow-green fluorescent beads from consecutive timeframes did not exceed 30 pixels (that is, 3.3 µm). Volume was calculated from the radius by assuming a perfectly spherical MP (that is, V = (4/3)πr3). We assessed the intensity of the MP on the original image as the average pixel intensities within an inner circle whose radius was half that of the radius of the MP (where the ‘full radius’ was determined using the Hough circle detector described above).

For ratiometric pH measurements, MPs were detected as described above at the lower 95th percentile channel. Intensities were measured on both images at the same coordinates. Background values were calculated as 2nd percentile across the whole z-projected image individually for each time step image and further subtracted from MP intensity values. As a final step, for each MP, intensities at the pH-sensitive wavelength (λ = 515 nm) were normalized to the corresponding pH-insensitive wavelength (λ = 455 nm). When using heterozygous expression, transfected cells were selected and cropped from the image manually on the basis of GFP expression.

Scratch assay. BMDMs were seeded in M-CSF-free complete DMEM on eight-well glass bottom µ-slide (ibidi, 80827) at 85% confluency minimally 24 h before the experiment. Cells were placed either in complete DMEM containing 150 ng ml−1 recombinant murine CSa (PeproTech, 315–40) or in a CsA-free DMEM. The cell monolayer was scratched with a 10 µl pipette tip and immediately placed on the microscope for imaging. Phase contrast imaging of the scratch and surrounding cells was performed on a Nikon Ti Eclipse microscope operated with NIS software in an 15 mm glass-bottom 24-well plate (MatTek) for 15 h on 20x air objective (Plan Apo NA 0.75, Nikon) with back-illuminated SCMOS camera (Prime95B, Photometrics) using Nikon Volume Contrast function. Final pixel size was 0.55 µm.

Data were analysed using custom code using Python (version 3.8). The first image in the time series (t = 0) was segmented using an inverse_Gaussian_gradient filter (scikit-image function skimage.feature.inverse_gaussian_gradient, parameters alpha = 100, sigma = 5 pixels), followed by thresholding the image below the 20th percentile of the intensity distribution (of the inverse filtered images). Then images were thresholded using skimage.filters.threshold_local function of scikit-image (parameters: block size 41, offset 10). Further, we performed morphological opening of the image (erosion followed by a dilation) using skimage morphology:opening function with disk footprint with 40 px radius following by two morphological reconstructions of the image (first dilation, then erosion, skimage.morphology:reconstruction function) to separate scratch area from surrounding cells. We detected the borders of the scratch on this image mask and used the findContours and drawContours functions in the OpenCV package (version 4.2.0) and selecting the longest detected contour. Using the same mask for the original scratch area, we detected and counted the number of cells at every following time step image by filtering the image with a sequence of difference of Gaussian (DoG) filters (scikit-image package, DoG function skimage.feature.blob dog) with minimum and maximum standard deviations of the Gaussian kernels used set to 5 and 30 pixels correspondingly and absolute lower bound for scale space maxima set to 0.02. Finally, we subtracted the number of cells detected on the scratch area at t = 0 from number of cells detected at every following timeframe and normalize this number to the original area of scratch (calculated in number of pixels and further converted to µm2).
Articles

Phagocytosis of *E. coli*. In order to determine whether TMEM206 is present in phagosomes, WT BMDM were incubated with green fluorescent *Escherichia coli* (E. coli K-12 strain) BioParticles, Alexa Fluor 488 conjugate, Thermo Fischer, E13231 diluted in complete DMEM to obtain a macrophages/E. coli ratio of 1/10. After 15 min of incubation, cells were washed twice with complete DMEM and incubated for additional 5 min with complete DMEM at 37 °C. Cells were then fixed with 4% PFA and stained for TMEM206 as described above.

Statistics and reproducibility. Statistical tests were performed using GraphPad Prism (v.7.03). Statistical test was chosen on the basis of data distribution normality, which was checked with Shapiro–Wilk test. Either unpaired two-tailed parametric t-test or two-tailed non-parametric Mann–Whitney test was performed for comparing two groups of data. For multiple comparisons, either one-way analysis of variance (ANOVA) (parametric) or Kruskal–Wallis (non-parametric) tests were used.

On average, 30 MPs were detected and successfully tracked through all timeframes per one round of imaging on the MatTek dish. One to five MatTek dishes were imaged per condition per different BMDM preparation. MP measurements (volume, fluorescent intensity or intensity ratio) were averaged first per dish and then per BMDM preparation, which formed one data point on the plot. For overexpression experiments, MP measurements (volume and fluorescent intensity) were averaged per cell and then per transfection round (within the same animal). Statistical comparison was done on averaged values per cropped image with transfected cell. Plots represent means ± s.e.m. n is animal number, and N is number of MPs.

No statistical methods were used to predetermine sample sizes. All western blot or immunofluorescence experiments were repeated at least two times independently with similar results being obtained. All MP shrinkage experiments were repeated at least three times independently with similar results.

Co-localization analysis. For co-localization analyses separate cells or single MPs were cropped in rectangles minimizing the surrounding background area. Pearson’s correlation coefficient above threshold was calculated using Fiji (bisection threshold regression). Pearson’s R was plotted for separate cells (each dot corresponds to one cell) or first averaged across MPs present within the same one cell and then plotted (each dot corresponds to an average of MPs per cell). Plots represent means ± s.e.m. n is number of analysed cells.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Source data are provided with this paper. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Code availability

The code is freely available on Github repository: mathematical model ([https://github.com/mzeziulia/MP_volume_modelling](https://github.com/mzeziulia/MP_volume_modelling)); MP detection and analysis ([https://github.com/mzeziulia/MP_detection_analysis](https://github.com/mzeziulia/MP_detection_analysis)); scratch assay analysis ([https://github.com/mzeziulia/Scratch_assay](https://github.com/mzeziulia/Scratch_assay)).

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Author contributions

M.Z. designed, performed and analysed MP live cell imaging and physiological assays and implemented numerical simulations of the mathematical model in Python. S.B.

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Competing interests

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Extended Data Fig. 1 | Specificity of custom-made antibodies against TMEM206. Western Blot of membrane preparations of WT and Tmem206 KO HEK cells to check for specificity of two custom-made rabbit anti-TMEM206 antibodies, one directed against the C-terminus of mouse TMEM206 (against the peptide sequence IKIRKRYLRRGATNHIS (a) and another directed against the extracellular loop (against the peptide sequence VKTKEEDGREAVEFRQET) (b). Both antibodies had been affinity-purified with the cognate peptide. While both antibodies recognized several unspecific bands, bands at the correct size were missing in KO samples. CT1-F1 antibody was used for the immunodetection of TMEM206 in BMDMs and HEK cells after antigen retrieval and proved to be specific as evident from loss of signal in Tmem206 KO cells (Fig. 2a, Extended Data Fig. 3a). Na/K-ATPase α1 subunit was used as loading control. 30 µg of membrane preparation were loaded per lane for each sample. Source unprocessed blots are available in source data.
Extended Data Fig. 2 | Expression patterns of TMEM206 and ClC-7 in primary bone marrow-derived macrophages. (a) Endogenous TMEM206 co-localizes with early endosomal rab5 and partially with late endosomal rab7. Pearson’s R calculated from 19 cells (Rab5), 27 cells (Rab7) from 2 independent experiments. (b) Expression of human TMEM206 C-terminally tagged with GFP in Tmem206−/− primary macrophages gives similar expression pattern as endogenous TMEM206 protein. (c) ClC-7 co-localizes with late endosome marker rab7. Pearson’s R calculated from 26 cells from 2 independent experiments. Mean ± s.e.m. Scale bars: 10 µm, 1 µm for enlargements. Source numerical data are available in source data.
Extended Data Fig. 3 | TMEM206 is present in early endosomes in HEK cells. (a) Immunofluorescence staining of endogenous TMEM206 in HEK WT cells. This staining is abolished in TMEM206 KO HEK cells. (b) Endogenous TMEM206 co-localizes with EEA1 and rab5, early endosomes markers, and partially with rab7, a marker of late endosomes. Scale bars: 10 μm, 1 μm for enlargements.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Generation of Tmem206−/− mouse lines and TMEM206 KD in HT-1080 cells. (a) Strategy for disrupting Tmem206 in mice using CRISPR-Cas9 genome editing technique. gRNAs (in red) g1 and g5, both targeting exon 2, coding for the N terminus of TMEM206, were injected together and led to a 62 bp deletion. Similarly, g1 or g2, also targeting exon 2, were injected together with g6 targeting exon 3 after the sequence coding for the first transmembrane domain, leading to a deletion of ≈10 kbp. These injections led to the generation of three different Tmem206 KO mouse lines called T6-1/5 (injection of g1 and g5), T6-1/6 (injection of g1 and g6) and T6-2/6 (injection of g2 and g6). (b) Sequencing of one of the founders of the T6-1/5 line. (c) Genotyping PCR of WT homozygous (+/+), heterozygous (+/-) and KO homozygous (-/-) mice from the T6-1/5 line using the genotyping primers (a, in green) flanking the exon 2. (d) Western blot analysis of TMEM206 expression in different tissues from WT and KO mice confirmed that TMEM206 is deleted in all 3 different KO lines. Key experiments were done with BMDMs from these three different lines to exclude possible off-target effects of sgRNAs. 30 µg protein of membrane preparation were loaded per lane for each sample. TMEM206 proteins were detected using the custom-made antibodies targeted against the extracellular loop. Na/K-ATPase α1 subunit was used as loading control. The WB done with animals from the T6-1/5 line, TMEM206 signals from different organs were obtained from different membranes but at the same exposure. This also holds true for the T6-1/6 line, except for the pancreas samples, which were obtained after shorter exposure time compared to the other organs. For the T6-2/6 line, all the signals come from the same membrane and exposure time, except for the pancreas samples, which were obtained after shorter exposure time compared to the other organs. (e) TMEM206 knock-down (KD) efficiency in HT-1080 cells compared to cells transfected with non-targeting siRNA (ctrl), quantified in Fiji, 30 µg of protein per lane, n=5 (independent transfections). Na/K ATPase is a loading control. TMEM206 protein was detected using custom-made antibody against the extracellular loop. Mean ± s.e.m. Source numerical data and unprocessed blots are available in source data.
Extended Data Fig. 5 | Disruption of Clcn2, Clcn3, Clcn4, Clcn5, Clcn7 and Lrrc8a did not affect macropinosome resolution. (a-e) For each genotype volume decrease (upper panels) and fluorescence intensity increase (lower panels) are not significantly different from WT (n=3 mice for Clcn2−/− (N=294) and Clcn3−/− (N=266), n=4, N=125 for WT); n=3 for Clcn4−/− (N=270) and WT (N=235); n=5, N=822 for Clcn5−/− and n=3, N=263 for WT; n=3 for Clcn7−/− (N=19) and WT (N=74); n=4, N=311 for Lrrc8a−/− and n=3, N=159 for WT. Clcn7−/− cells were selected by YFP expression. Plot of mean ± s.e.m. (shown as bands), averaging means from individual mice. (f) Western blot showing decreased expression of LRRC8A in Cx3cr1-CreERT2; Lrrc8alox/lox BMDMs after tamoxifen (Tam) induction (top panel). Actin (bottom panel) was used as loading control. 10 µg of total protein were loaded per sample. Source numerical data and unprocessed blots are available in source data.
Extended Data Fig. 6 | Initial formation of macropinosomes and phagosomes is not affected by Tmem206 knock-out. (a) No effect of ion replacement or Tmem206 disruption on initial formation of macropinosomes. Each dot represents the mean number of detected macropinosomes per cell from BMDM preparation from one individual mouse, with at least 100 BMDMs evaluated per mouse. n=20 (WT); n=10 (Tmem206−/−); n=9 (low Cl−, 9 mM); n=7 (low Na+, nominally 0 mM). No significant difference between any conditions (Kruskal-Wallis test with Dunn’s multiple comparison). p>0.9999 (WT – Tmem206−/−), p>0.9999 (WT – low Cl−), p=0.3264 (WT – low Na+).

(b) BMDMs were incubated with killed fluorescent E. coli (green) and fixed after 5 and 15 min of incubation. TMEM206 was detected by immunostaining (red). Vesicles containing one or more bacteria were decorated with TMEM206-positive dots. Scale bars: 5 µm, 2 µm for enlargements.

(c) Number of beads of 3- or 6-µm diameter phagocytosed by WT and Tmem206−/− macrophages, incubated with or without cytochalasin D (10 µM), an actin inhibitor. Two sizes (3- and 6-µm diameter) of beads were tested since disruption of Trpml1 or BK channels has been reported to differentially affect only larger beads. Similar to our observation for the formation of macropinosomes, disruption of Tmem206 lacked an effect on the uptake of either size class of beads. n=4 mice per condition. Unpaired two-tailed t test. Mean ± s.e.m. Source numerical data are available in source data.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Localization of LRRC8A in BMDMs. (a) *Lrrc8a*3xHA/3xHA mice that express a LRRC8 protein C-terminally tagged with 3 copies of the HA-epitope from the native genomic locus were used to determine the subcellular localization of the essential VRAC subunit LRRC8A. Anti-HA antibodies detected the protein in the plasma membrane of *Lrrc8a*3xHA/3xHA BMDMs but not in WT control macrophages. (b) LRRC8A is present at the plasma membrane of *Lrrc8a*3xHA/3xHA BMDMs. There is no significant co-localization with endolysosomal markers rab5 (early endosomes), rab7 (late endosomes) or LAMP1 (lysosomes). Pearson’s R calculated from 18 cells (Rab5), 20 cells (Rab7), 16 cells (LAMP1). (c) 4 min after M-CSF addition, LRRC8A can be detected in early macropinosomes (co-stained by rab5) in ≈5-10% of cells, but not in rab7-positive mature macropinosomes. All small panels represent magnified macropinosomes or regions of interest indicated by white arrowheads. Pearson’s R calculated from 15 cells (Rab5, 4min), 10 cells (Rab7, 4min), 5 cells (Rab5, 7min), 10 cells (Rab7, 7min). Mean ± s.e.m. Scale bars: 10 μm and 1 μm for the magnification panels. Source numerical data are available in source data.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Localization of LRRC8D in BMDMs. (a) Localization of LRRC8D used BMDMs from Lrrc8d<sup>tdTomato/tdTomato</sup> knock-in mice<sup>55</sup> that were stained with an anti-RFP antibody. No fluorescence signal was detected in WT macrophages (negative control). (b) No significant co-localization of LRRC8D in BMDMs with early endosomal rab5, late endosomal rab7 or LAMP1 (lysosomes) was observed. Pearson’s R calculated from 17 cells (Rab5), 17 cells (LAMP1), 14 cells (Rab7). (c) LRRC8D could not be detected in macropinosomes (detected by rab5 and rab7 stainings), neither at 4 nor at 7 min after M-CSF stimulation. All small panels represent magnified macropinosomes or regions of interest indicated in left panels by white arrowheads. Pearson’s R calculated from 16 cells (Rab5, 4min), 11 cells (Rab7, 4min), 11 cells (Rab5, 7min), 9 cells (Rab7, 7min). (d) Localization of LRRC8A/LRRC8D heteromers in transfected cells. Untagged-LRRC8A and tdTomato-tagged LRRC8D were co-transfected (ratio 1:1) in HeLa WT cells to determine the subcellular localization of LRRC8A/LRRC8D heteromers. Note that LRRC8A needs LRRC8D to leave the endoplasmic reticulum and for its transport to the plasma membrane<sup>11</sup>. In contrast to work by others<sup>14</sup>, we detected LRRC8D almost exclusively at the plasma membrane but not in lysosomes (consistent with our results on BMDMs, Extended Data Figs. 7 and 8). 4 different cells expressing both proteins are shown. Mean ± s.e.m. Scale bars: 10 µm and 1 µm for enlargements. Source numerical data are available in source data.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Localization of the α3 V-ATPase subunit in BMDMs. (a) The V-ATPase α3 subunit shows significant co-localization with lysosomal/late endosomal LAMP1 and late endosomal rab7, but not with early endosomal rab5 in unstimulated BMDMs. Pearson’s R calculated from 12 cells (EEA1), 8 cells (Rab7), 8 cells (LAMP1). (b, c) Upon exposure to M-CSF, the α3 subunit was detected in rab7-positive macropinosomes after 7 min (c), but not after 4 min (b). It was not found in rab5-positive MPs. Pearson’s R calculated from 12 cells (EEA1, 4min), 18 cells (Rab7, 4min), 11 cells (EEA1, 7min), 13 cells (Rab7, 7min). All small panels represent enlarged images of macropinosomes or regions of interest indicated by white arrowheads. Mean ± s.e.m. Scale bars: 10 μm and 1 μm for enlargements. Source numerical data are available in source data.
Extended Data Fig. 10 | Luminal pH of macropinosomes. (a) Calibration of 70 kDa Oregon Green dye with Boltzmann sigmoidal fit (red line), n=1 (BMDMs prepared from one animal), ≈ 150 cells per replicate (each dot is a new field of view, 4 fields in total that belong to 2 different imaging dishes). pH<sub>½</sub><sup>calculated</sup><sub>calculated</sub> = 4.7 calculated from calibration curve is similar to provided by manufacturer pK<sub>a</sub><sup>provided</sup><sub>provided</sub> = 4.7. (b–c) Neither disruption of Clcn4 (b) nor of Clcn5 (c) significantly changed macropinosomal pH when measured from 5 min after M-CSF application onwards. n=3 for Clcn4<sup>−/−</sup> (N=337) and corresponding WT (N=118); n=5, N=389 for Clcn5<sup>−/−</sup> and n=2, N=141 for corresponding WT. Plot of mean ± s.e.m. (shown as bands), averaging means from individual mice. Source numerical data are available in source data.
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- □ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
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- □ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Live imaging of the macropinosomes was performed using Nikon spinning disc microscope (Yokogawa spinning disk 695 CSU-X1) operated with NIS software (version 5.02.01 Build 1270). Scratch assay was performed using Nikon Ti Eclipse microscope operated with NIS software (version 5.21.03 Build 1481). For immunocytochemistry pictures, the confocal microscope LSM880 (Zeiss, Zen Blue software 2.3) was used. Electrophysiological recordings were performed using an EPC-10 USB patch-clamp amplifier and PatchMaster software (HEKA Elektronik, version 2x90.3). For growth assay absorbance was measured with absorbance plate reader (ASYS Hitachi).

Data analysis
All microscopy images were analysed using Fiji 2.0/2.1 versions. Custom data analysis code was written using Python 3.8 using OpenCV (version 4.2.0) and scikit-image (version 0.16.2) packages. Plotting and statistical analyses were performed with GraphPad Prism 7.03.

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Data is freely available from authors upon request. Code is freely available on Github repository: mathematical model [https://github.com/mzecuili]
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample sizes were not predetermined with statistical means but were based on standard numbers in the field. Each experiment based on live cell microscopy was performed on at least 3 animals resulting in 10-800 vesicles analysed per condition. For immunocytochemistry, an average 15 cells per condition were analysed. Western Blot analyses were performed on 2-3 different animals. For growth assay 3 different knock-out cell lines generated with 2 different sgRNAs were used, experiments were repeated 3 times, each measurement in 4 technical replicates. For scratch assay 13 pairs of animals were used.

Data exclusions

No data were excluded.

Replication

All experiments were repeated independently on minimum 2 different animals with the same outcome, all obtained quantitative data is shown in figures. Key experiments based on live cell imaging were performed on 3 different TEM206 knock out mouse lines. Experiments using cell lines were repeated independently on cells with different passage number with the same outcome. Growth assay was performed 3 times on 3 different knock-out cell lines generated with 2 different sgRNAs with the same outcome.

Randomization

No animal randomization was done because mice are assigned to their group based on genotype. Microscopic fields were randomly chosen when possible.

Blinding

Genotypes of animals for scratch assay and genotypes of MIA PaCa-2 cells for growth assay were blinded. No blinding was applied for macropinosome shrinkage assay since it was logistically impossible but microscopic fields were randomly chosen.

Key experiments based on live cell imaging were analyzed automatically by a custom code, minimizing potential analysis bias.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
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| Eukaryotic cell lines | Flow cytometry |
| Palaeontology and archaeology | MRI-based neuroimaging |
| Animals and other organisms | |
| Human research participants | |
| Clinical data | |
| Dual use research of concern | |

Antibodies

The following antibodies were used:

- anti-LAMP1 (rat, BD Pharmingen, 553792)
- anti-EA1 (mouse, Abcam, ab70571)
- anti-EA1 (sheep, R&D Systems, AF8047)
- anti-Rab5A (mouse, Cell Signaling, 46449)
- anti-Rab5 (mouse, BD Bioscience, BD610725)
- anti-Rab7 (mouse, SCBT, sc-376362)
- anti-GFP (chicken, Avexis lab, GFP-1020)
- anti-HA (rabbit, Cell Signaling, 3724)
- anti-RFP (rabbit, Rockland, 600-401-379)
- anti-α1 Na/K ATPase clone C464.6 (mouse, Millipore, 05–369)
- anti-actin (mouse, Sigma, A2228)
- anti-actin (rabbit, Sigma, A2066)
Validation

All commercially available antibodies were validated by manufacturers and used in published papers [see manufacturers’ websites for immunohistochemistry on western blot examples].

- anti-LAMP1 antibody (rat, BD Pharmingen, 553792) was validated in https://www.citeab.com/antibodies/2410022-553792-bd-pharmingen-purified-rat-anti-mouse-cd107a
- anti-EA1 (mouse, Abcam, ab70521) was validated in https://www.abcam.com/ea1-antibody-1g11-early-endosome-marker-ab70521.html
- anti-EA1 (sheep, R&D Systems, AF8047) was validated in https://www.rndsystems.com/products/human-mouse-rat-ea1-antibody_af8047
- anti-Rab5A (mouse, Cell Signaling, 46449) was validated in https://www.cellsignal.com/products/primary-antibodies/rab5a-e6n8s-mouse-mab/46449
- anti-Rab5 (mouse, BD Bioscience, BD610725) was validated in https://wwwbdbiosciencesen-de/products/reagents/microscopy-imaging-reagents/immunofluorescence-reagents/purified-mouse-anti-rab5.610725
- anti-GFP (chicken, Aves lab, GFP-1020) was validated in https://www.aveslabs.com/products/anti-green-fluorescent-protein-antibody-gfp
- anti-HA (rabbit, Cell Signaling, 3724) was validated in https://www.cellsignal.com/products/primary-antibodies/ha-tag-c294-rabbit-mab/3724
- anti-RFP (rabbit, Rockland, 600-401-379) was validated in https://www.rockland.com/categories/primary-antibodies/rfp-antibody-pre-adsorbed-600-401-379/
- anti-a Na/K ATPase clone C464.6 (mouse, Millipore, 05-369) was validated in https://www.merckmillipore.de/de/de/product/Anti-Na-K-ATPase-1-Antibody-clone-C464.6_MN_NF-05-369?referrerURL=https%3A%2F%2Fwww.google.com%2F
- anti-actin (mouse, Sigma, A2228) was validated in https://www.sigmaaldrich.com/DE/en/product/sigma/a2228
- anti-actin (rabbit, Sigma, A2666) was validated in https://www.sigmaaldrich.com/DE/en/product/sigma/a2666

Newly generated custom-made TEM206 antibodies were validated with Western Blot analysis and immunocytochemistry using HEK TEM206 KO cells and tissues/cells from different TEM206 knock-out mouse lines. All other custom-made antibodies (CIC-2, CIC-3, CIC-4, CIC-5, CIC-6, CIC-7, LRRC8A and a3 subunit of V-ATPase) were previously produced in our lab and were validated according to the field’s highest standards by Western Blot analysis and immunohistochemistry on tissues from respective knock-out mouse lines [see methods and reference sections in the manuscript].

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Wild-type HEK293, HeLa, HT-1080 and MIA PaCa-2 are from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany.

Authentication
Parental cells of all above mentioned cells lines were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany.

Mycoplasma contamination
In all experiments cells were mycoplasma negative.

Commonly misidentified lines
No commonly misidentified lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
C57BL/6 Mice (Mus musculus) aged of 8-22 weeks (8-16 weeks for BMDMs preparation) and from both sexes were used in this study. The following lines were used: B6;Lrcc8atm2c(EUCOMM)Hmgu-em2(HA)Tj
B6;Lrcc8atm1[tomato;loxP]Tj
B6;129/Sv-Clcn2tm1Tj
B6;129/Sv-Clcn3tm1Tj
B6;129/Sv-Clcn4tm1Tj
B6;129/Sv-Clcn5tm1Tj
B6;129/Sv-Clcn7tm1.1Tj x C3cr1CreER x ROSA26flloxSTOP-YFP
B6;Lrcc8atm2a(EUCOMM)Hmgx x C3cr1CreER

and the 3 newly generated TEM206 knock-out lines (as described in the methods section).

Wild animals
No wild animals were used.
| Field-collected samples | No samples were collected from the field. |
|-------------------------|------------------------------------------|
| Ethics oversight        | All animal experiments and the generation of new mouse lines were approved by Berlin authorities (LAGeSo, G0005/19 licence). |

Note that full information on the approval of the study protocol must also be provided in the manuscript.