IP$_3$ Receptors Preferentially Associate with ER-Lysosome Contact Sites and Selectively Deliver Ca$^{2+}$ to Lysosomes

Graphical Abstract

Highlights

- IP$_3$ receptors (IP$_3$Rs) selectively deliver Ca$^{2+}$ to lysosomes
- Lysosomes associate preferentially with clusters of IP$_3$Rs in ER membranes
- Low lysosomal pH maintains the IP$_3$R-lysosome contacts required for Ca$^{2+}$ uptake
- ER and its Ca$^{2+}$ channels deliver Ca$^{2+}$ to low-affinity lysosomal transporters

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In Brief

Ca$^{2+}$ exchanges between ER and lysosomes regulate cytosolic Ca$^{2+}$ signals and lysosome behavior. Atakpa et al. show that clusters of IP$_3$ receptors populate ER-lysosome contact sites and facilitate local delivery of Ca$^{2+}$ from the ER to lysosomes.
IP₃ Receptors Preferentially Associate with ER-Lysosome Contact Sites and Selectively Deliver Ca²⁺ to Lysosomes

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SUMMARY

Inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃R) allow extracellular stimuli to redistribute Ca²⁺ from the ER to cytosol or other organelles. We show, using small interfering RNA (siRNA) and vacuolar H⁺-ATPase (V-ATPase) inhibitors, that lysosomes sequester Ca²⁺ released by all IP₃R subtypes, but not Ca²⁺ entering cells through store-operated Ca²⁺ entry (SOCE). A low-affinity Ca²⁺ sensor targeted to lysosomal membranes reports large, local increases in cytosolic Ca²⁺ during IP₃-evoked Ca²⁺ release, but not during SOCE. Most lysosomes associate with endoplasmic reticulum (ER) and dwell at regions populated by IP₃R clusters, but IP₃Rs do not assemble ER-lysosome contacts. Increasing lysosomal pH does not immediately prevent Ca²⁺ uptake, but it causes lysosomes to slowly redistribute and enlarge, reduces their association with IP₃Rs, and disrupts Ca²⁺ exchange with ER. In a “piston-like” fashion, ER concentrates cytosolic Ca²⁺ and delivers it, through large-conductance IP₃Rs, to a low-affinity lysosomal uptake system. The involvement of IP₃Rs allows extracellular stimuli to regulate Ca²⁺ exchange between the ER and lysosomes.

INTRODUCTION

Increases in cytosolic free Ca²⁺ concentration ([Ca²⁺]₀) regulate the activities of all cells, allowing them to respond to internal and extracellular signals. Most Ca²⁺ signals are evoked by opening of Ca²⁺-permeable channels within the plasma membrane (PM) or the membranes of intracellular organelles, usually the endoplasmic reticulum (ER). In non-excitatory cells, extracellular stimuli typically evoke Ca²⁺ signals by stimulating phospholipase C (PLC), which catalyzes formation of inositol 1,4,5-trisphosphate (IP₃), which in turn triggers a series of events that leads to the release of Ca²⁺ from intracellular stores, including the ER. IP₃Rs selectively deliver Ca²⁺ to lysosomes. Many long-lived signals, to ER, selectively sequester Ca²⁺ released by IP₃Rs, and this then regulates mitochondrial behavior (Rizzuto et al., 2012). Ca²⁺ released through IP₃Rs or ryanodine receptors (RyR) adjacent to the PM regulates membrane potential by selectively activating Ca²⁺-sensitive Cl⁻ or K⁺ channels (Courjaret et al., 2017; Nelson et al., 1995). Depolarization of cardiac muscle opens voltage-gated Ca²⁺ channels, and the resulting local Ca²⁺ signals are amplified by Ca²⁺-induced Ca²⁺ release (CICR) from RyRs (Rios, 2018). Loss of Ca²⁺ from the ER stimulates Ca²⁺ channels in the PM, and the resulting store-operated Ca²⁺ entry (SOCE) selectively regulates adenyl cyclase, nitric oxide synthase, and nuclear factor of activated T cells (Prakiya and Lewis, 2015). For each of these examples, and many others, the specificity of the Ca²⁺ signal is conferred by having a channel deliver Ca²⁺ at a high local concentration to closely apposed target proteins.

Lysosomes can also sequester Ca²⁺ and they express channels, including TRPML (transient receptor potential mucolipin), TPC2 (two-pore channel 2), and ATP-regulated P2X4 receptors, that allow Ca²⁺ release (Morgan et al., 2011). Here, too, crosstalk with the ER is important, and it is facilitated by membrane contact sites (MCSs) between lysosomes and ER, stabilized by scaffold proteins (Alpy et al., 2013; Eden, 2016; Friedman et al., 2013; Kilpatrick et al., 2017). The cytosolic Ca²⁺ signals evoked by TRPML or TPC2 can be amplified by CICR through IP₃Rs or RyRs in closely apposed ER (Galione, 2015; Morgan et al., 2011; Patel et al., 2010). Conversely, Ca²⁺ released by ER channels can be rapidly sequestered by lysosomes. This sequestration attenuates cytosolic Ca²⁺ signals evoked by IP₃Rs (López Sanjurjo et al., 2013) and, by loading lysosomes with Ca²⁺, primes TPC2 to respond (Morgan et al., 2013), controls fusion and fission within endolysosomal pathways (Ruas et al., 2010), and regulates autophagy and lysosomal biogenesis through calsenilin activated by TRPML-mediated Ca²⁺ release (Medina et al., 2015). Using pharmacological inhibitors that disrupt lysosomes, perturb their morphology, or block their ability to sequester H⁺, we showed previously that the increase in [Ca²⁺]₀ evoked by IP₃Rs was exaggerated when lysosomes were disrupted, but SOCE-evoked Ca²⁺ signals were unaffected (López Sanjurjo et al., 2013, 2014). We suggested that lysosomes selectively sequester Ca²⁺ released by IP₃Rs, while ignoring Ca²⁺ entering cells through SOCE.

Using targeted low-affinity Ca²⁺ sensors, we now show that IP₃Rs selectively deliver Ca²⁺ to lysosomes. Many long-lived contacts between ER and lysosomes are populated by small
The vacuolar H+-ATPase (V-ATPase) maintains the luminal pH of lysosomes at about 4.5. Treatment of HEK cells with concanamycin A (CcA), a more selective inhibitor of the V-ATPase than bafilomycin A₁ (Dröse et al., 1993), dissipated the lysosomal pH gradient (Figure 1A) and modestly increased the basal [Ca²⁺]₀ (Figures 1B and 1C). The peak increase in [Ca²⁺]₀ evoked by CCh (D). Results show paired individual values (each from three determinations) and the mean (n = 7, line). *p < 0.05, paired Student’s t test.

## RESULTS

### Lysosemes Selectively Sequester Ca²⁺ Released by IP₃Rs

The vacuolar H⁺-ATPase (V-ATPase) maintains the luminal pH of lysosomes at about 4.5. Treatment of HEK cells with concanamycin A (CcA), a more selective inhibitor of the V-ATPase than bafilomycin A₁ (Dröse et al., 1993), dissipated the lysosomal pH gradient (Figure 1A) and modestly increased the basal [Ca²⁺]₀ (Figures 1B and 1C). The peak increase in [Ca²⁺]₀ evoked by CCh (D). Results show paired individual values (each from three determinations) and the mean (n = 7, line). *p < 0.05, paired Student’s t test.

(A) Bright-field and wide-field fluorescence images of HEK cells loaded with LysoTracker Red (100 nM, 10 min) with or without CcA (1 μM, 1 hr). Images are typical of three experiments.

(B) Fluorescence-loaded HEK cells were treated with CcA (1 μM, 1 hr) in HBS before addition of 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (2.5 mM) to chelate extracellular Ca²⁺ and then CCh (1 mM) to stimulate IP₃ formation. Typical traces show mean ± SD from three wells in one experiment.

(C) and (D) Summary results show effects of CcA on basal [Ca²⁺]₀ (C) and peak increase in [Ca²⁺]₀ (Δ[Ca²⁺]₀) evoked by CCh (D). Results show paired individual values (each from three determinations) and the mean (n = 7, line). *p < 0.05, paired Student’s t test.

(E) Expression of mRNA for ATP6V0C relative to GAPDH in cells treated with non-silencing siRNA (NS) or siRNA for ATP6V0C. Mean ± SEM, n = 6. *p < 0.05, paired Student’s t test.

(F) TIRFM images show effects of siRNAs in HEK cells expressing TPC2-GFP or stained with LysoTracker Red (100 nM, 10 min). Images are typical of three experiments.

(G) Effects of siRNA on basal [Ca²⁺]₀ (n = 6, each with three determinations).

(H) Effects of siRNA on Δ[Ca²⁺]₀ evoked by CCh alone or after CcA (1 μM, 1 hr) (mean ± SEM, n = 5, each with three determinations). *p < 0.05, two-way ANOVA with Bonferroni test.

(I) Results and Figure S1 demonstrate that lysosomes (LY) selectively sequester Ca²⁺ released from ER through IP₃Rs, but not Ca²⁺ entering the cell through SOCE.

See also Figure S1.
Figure 2. IP₃Rs Selectively Deliver Ca²⁺ to Some Lysosomes

(A) Wide-field fluorescence images of HeLa cells expressing TPC2-RFP with Ly-GG or Cy-GG.

(B and C) Recordings from HeLa cells expressing Cy-GG (B) or Ly-GG (C) showing responses to histamine (100 μM) in Ca²⁺-free HBS and then ionomycin (10 μM) with 2 mM CaCl₂ (to saturate the sensor). Results (F/Fₘₐₓ, where Fₘₐₓ is response after ionomycin) show responses of a single tracked lysosome (C) or a similarly sized cytosolic region of interest (ROI) (B) (see Video S1).

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effects of CcA on CCh-evoked Ca\(^{2+}\) signals are mediated by inhibition of the V-ATPase. The results are important because bafilomycin A\(_1\) and CcA have additional effects, which at higher concentrations include inhibition of the P-type ATPases that transport Ca\(^{2+}\) across the PM and ER membranes (Dröse et al., 1993). We conclude that inhibition of the V-ATPase potentiates the increase in [Ca\(^{2+}\)]\(_c\), evoked by Ca\(^{2+}\) release through IP\(_3\)Rs.

The sustained response to CCh, and to most stimuli that activate PLC, requires Ca\(^{2+}\) entry across the PM through SOCE, which is stimulated when IP\(_3\) causes loss of Ca\(^{2+}\) from the ER (López Sanjurjo et al., 2014; Prakriya and Lewis, 2015). In cells pretreated with thapsigargin in Ca\(^{2+}\)-free HEPES-buffered saline (HBS) to inhibit SERCA and deplete the ER of Ca\(^{2+}\), restoration of extracellular Ca\(^{2+}\) caused a sustained increase in [Ca\(^{2+}\)]\(_c\), reflecting the activity of SOCE (Figure S1C). Although the global increase in [Ca\(^{2+}\)]\(_c\) resulting from thapsigargin-evoked SOCE was comparable with the increase after CCh-evoked Ca\(^{2+}\) release (Figures 1B and S1C), SOCE signals were unaffected by CcA, bafilomycin A\(_1\), or knockdown of the V-ATPase (Figures S1D–S1F).

These results extend previous observations (López Sanjurjo et al., 2013) by demonstrating that dissipating the lysosomal pH gradient, using siRNA to the V-ATPase or pharmacological inhibitors, exaggerates cytosolic Ca\(^{2+}\) signals evoked by IP\(_3\)Rs, but not those evoked by SOCE (Figure 1I).

### IP\(_3\)Rs Selectively Deliver Ca\(^{2+}\) to Lysosomes

The conclusion that Ca\(^{2+}\) released from the ER is selectively accumulated by lysosomes has so far been inferred from exaggerated increases in [Ca\(^{2+}\)]\(_c\) after perturbing lysosomes (Figures 1 and S1A–S1I). We attempted to provide direct evidence, free of these perturbations, using a low-affinity Ca\(^{2+}\) sensor (G-GECO1.2, K\(_{50}^D = 1.2 \mu M\) (Zhao et al., 2011) targeted to the cytosolic surface of lysosomes by attaching it to LAMP1 (Ly-GG). For comparison with these measurements of [Ca\(^{2+}\)]\(_c\) near lysosomal membranes, we used the same sensor expressed in the cytosol (Cy-GG) to record global increases in [Ca\(^{2+}\)]\(_c\) (Video S1). HeLa cells, in which histamine stimulates IP\(_3\) formation and Ca\(^{2+}\) release from the ER (Thillaiappan et al., 2017), were used for these experiments because they are better suited for imaging organelles. In HeLa cells, just as in HEK cells, IP\(_3\)-evoked increases in [Ca\(^{2+}\)]\(_c\) were potentiated by bafilomycin A\(_1\), whereas SOCE-evoked Ca\(^{2+}\) signals were not (Figures S1G–S1I). Because the peak [Ca\(^{2+}\)]\(_c\) after histamine stimulation does not exceed ~360 nM (Figure S1G), Ly-GG and Cy-GG selectively report local increases in [Ca\(^{2+}\)]\(_c\), in HeLa cells.

Ly-GG co-localized with LAMP1-mCh (R\(_{\text{coloc}} = 0.93 \pm 0.02\), n = 3; R\(_{\text{coloc}}\) is Pearson’s correlation coefficient) and with the lysosomal channel, TPC2-RFP (R\(_{\text{coloc}} = 0.86 \pm 0.09\), n = 3) (Figure 2A). In unstimulated cells, [Ca\(^{2+}\)]\(_c\) (reported as F/F\(_{\text{max}}\)) was similar when detected with Ly-GG or with Cy-GG (Figures 2B–2D). This confirms that the Ca\(^{2+}\)-affinity of the sensor was unaffected by differential targeting. Cells expressing the Ca\(^{2+}\) sensor and incubated in Ca\(^{2+}\)-free HBS were stimulated with histamine to evoke IP\(_3\) formation. Fluorescence was recorded from either single-tracked lysosomes (Ly-GG) or from comparable areas of cytosol (Cy-GG). Histamine caused a transient increase in Cy-GG fluorescence and a larger transient increase in Ly-GG fluorescence; the latter often came close to saturating the sensor (Figures 2C and 2E). From the distribution of fluorescence intensity changes of Ly-GG from 83 tracked lysosomes, 41% of responses overlapped those recorded from Cy-GG, but the remaining 59% of lysosomes responded with much larger fluorescence changes (Figures 2F and 2G).

There was no difference in the average speed of lysosomes responding to histamine with large (F/F\(_{\text{max}}\) > 0.6; speed = 0.45 ± 0.17 \mu m/s, n = 51) or cytosol-like responses (F/F\(_{\text{max}}\) < 0.6; 0.34 ± 0.22 \mu m/s, n = 32; p = 0.60). Similar analyses of SOCE revealed no disparity in the responses of Cy-GG and Ly-GG; both sensors reported similar increases in [Ca\(^{2+}\)]\(_c\) (Figure 2H).

We considered whether near-lysosome Ca\(^{2+}\) signals might be due to lysosomal Ca\(^{2+}\) channels, rather than to juxtaposed IP\(_3\)Rs. H\(_1\) receptors activated by histamine can, for example, stimulate accumulation of NAADP, which evokes Ca\(^{2+}\) release through TPC2 (Galione, 2015). However, two lines of evidence show that Ca\(^{2+}\) release through IP\(_3\)Rs is required for the near-lysosome Ca\(^{2+}\) signals. First, U73122, an inhibitor of PLC, abolished the histamine-evoked increases in [Ca\(^{2+}\)]\(_c\), whereas the inactive analog, U73343, did not (Figure S1J). U73122 also abolished histamine-evoked Ly-GG signals (Figures 2L and S1K). Second, direct activation of IP\(_3\)Rs by photolysis of caged IP\(_3\) (ci-IP\(_3\)) also caused increases in [Ca\(^{2+}\)]\(_c\) that were larger at the lysosome surface than in bulk cytosol (Figures 2I and 2J). Furthermore, whereas U73122 abolished Ly-GG responses to histamine, it had no effect on responses to photolysis of ci-IP\(_3\) (Figures 2L, S1K, and S1L), consistent with the effects of U73122 on histamine-evoked Ca\(^{2+}\) signals arising from inhibition of PLC.

(D and E) Summary results (mean ± SEM) show basal fluorescence (D) (3 experiments with 198 ROIs for Cy-GG and 83 tracks for Ly-GG) and peak fluorescence signals evoked by histamine (E) for Cy-GG (3 experiments with 198 ROIs) and Ly-GG (4 experiments with 83 lysosome tracks). *p < 0.05, Student’s t test.

(F and G) Distribution of peak F/F\(_{\text{max}}\) values for Cy-GG (F) and Ly-GG (G) in cells stimulated with histamine. Distribution of Ly-GG fluorescence values is significantly different from a normal distribution (Kolmogorov-Smirnov normality test, p = 0.0018), whereas Cy-GG fluorescence is consistent with a normal distribution (p > 0.1).

(H) Peak responses from tracked regions for Cy-GG and Ly-GG for cells in which SOCE was evoked by restoration of extracellular Ca\(^{2+}\) (2 mM) to cells treated with thapsigargin (1 \mu M, 15 min) in Ca\(^{2+}\)-free HBS. Mean ± SEM from at least three experiments (30 tracks for Ly-GG and 45 ROIs for Cy-GG).

(I and J) Distribution of peak F/F\(_{\text{max}}\) values for Cy-GG (I) and Ly-GG (J) after photolysis of ci-IP\(_3\) in Ca\(^{2+}\)-free HBS (n = 59 ROIs from 4 dishes for Cy-GG and 49 tracks from 3 dishes for Ly-GG).

(K) Summary results (mean ± SEM). *p < 0.05, Student’s t test.

(L) Effects of U73122 (10 \mu M, 20 min) on peak Ly-GG signals evoked by histamine (100 \mu M) or photolysis of ci-IP\(_3\). Mean ± SEM, n = 3–4. *p < 0.05, Student’s t test, relative to control.

See also Figure S1 and Video S1.
These results show that during Ca\(^{2+}\) release from IP\(_3\)Rs, about 60% of lysosomes experience a much larger increase in \([Ca^{2+}]_c\) than the global increase. SOCE, by contrast, does not deliver Ca\(^{2+}\) to lysosomes. We conclude that Ca\(^{2+}\) is selectively delivered to lysosomes by IP\(_3\)Rs, but not SOCE (Figure 1I).

**Lysosomes Sequester Ca\(^{2+}\) Released through All Three IP\(_3\)R Subtypes**

The Ca\(^{2+}\) signals evoked by histamine in HeLa cells or by CCh in HEK cells are initiated by IP\(_3\)Rs, consistent with the lack of response to CCh in HEK cells without IP\(_3\)R (Figure S1M). The HEK cells used express all three IP\(_3\)R subtypes (IP\(_3\)R3 > IP\(_3\)R1 > IP\(_3\)R2) (Mataragka and Taylor, 2018). We also used HEK cells where genes for one or two of the three IP\(_3\)R subtypes were disrupted (Alzayady et al., 2016) to establish whether any IP\(_3\)R subtype selectively presents Ca\(^{2+}\) to lysosomes. There was no significant difference in the basal \([Ca^{2+}]_c\) between the seven cell lines examined, nor did bafilomycin A\(_1\) affect basal \([Ca^{2+}]_c\) in any of the cells (Figure 3A). There were some unexpected differences in the amplitudes of the increase in \([Ca^{2+}]_c\) evoked by a maximal concentration of CCh (1 mM) between wild-type cells (Figure S1B) and cells lacking one or more IP\(_3\)R subtypes, with some of the latter giving larger signals than wild-type cells (Figures 3B–3G). We have not explored this further; it may arise from changes in expression of M\(_3\) receptors or downstream signaling proteins during selection of cell lines. However, the effects of bafilomycin A\(_1\) were similar in wild-type HEK cells and in cells lacking any one or two of the native IP\(_3\)R subtypes, irrespective of the amplitude of the CCh-evoked Ca\(^{2+}\) signals (Figures 3B–3G). In each case, bafilomycin A\(_1\) caused the increase in \([Ca^{2+}]_c\) evoked by CCh to increase by about 20%–30% (Figure 3H).

These results demonstrate that all three IP\(_3\)R subtypes can deliver Ca\(^{2+}\) to lysosomes.

**IP\(_3\)Rs and Lysosomes Preferentially Associate**

In COS-7 cells, most lysosomes detected near the PM by total internal reflection fluorescence microscopy (TIRFM) are associated with ER, and lysosomes maintain these associations as they move (López Sanjurjo et al., 2013). This is consistent with evidence from other cells showing that many lysosomes form dynamic MCSs with the ER (Friedman et al., 2013; Garrity et al., 2016; Kilpatrick et al., 2017). There is a similar relationship between the ER and lysosomes in HeLa cells (Figure 4A; Videos S2 and S3). We used HeLa cells in which endogenous IP\(_3\)R1 had been tagged by gene editing with EGFP (EGFP-IP\(_3\)R1-HeLa cells) (Thillaiappan et al., 2017) to explore dynamic relationships...
Figure 4. IP₃Rs Associate with ER-Lysosome Contacts

(A) TIRFM images of HeLa cell expressing mTurquoise-ER and LAMP1-mCherry showing that most lysosomes associate with ER and maintain contact as they move (yellow arrows). Images are shown at 8.4 s intervals (see Video S2).

(B) TIRFM images of EGFP-IP₃R1-HeLa cell expressing markers for lysosomes (mTurquoise-LAMP1) and ER (mCherry-ER). Merged image shows overlay of EGFP-IP₃R1 and lysosomes.

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between lysosomes and IP3Rs; mTurquoise-LAMP1 and mCherry-ER were used to identify lysosomes and ER using TIRFM (Figure 4B; Videos S4, S5, S6, S7, and S8). Within ER membranes, native IP3Rs form small clusters, or puncta, that include an average of eight tetrameric IP3Rs (Thillaiappan et al., 2017). Most lysosomes were mobile, but they often paused, sometimes for tens of seconds, at IP3R puncta (Figure 4C). This behavior is best illustrated as time series, or kymograms, showing the distribution of the ER, IP3R puncta, and lysosomes (Figures 4D, 4E, and S2–S4). We observed examples of lysosomes dwelling at immobile IP3R puncta, but pausing only briefly at immediately adjacent ER (Figures 4D, 4E, and S2D; Video S4). Some lysosomes remained with an IP3R punctum for tens of seconds before rapidly crossing intervening ER, and then dwelling at another immobile IP3R punctum (Figures S2A and S2D; Videos S4 and S5). In other cases, an IP3R punctum and a lysosome collided, and the pair stayed together as they moved or parked (Figures S2B and S2C; Video S6). We also observed two lysosomes associated with the same IP3R punctum before one lysosome departed to associate with a different IP3R punctum (Figure S3A). Finally, we observed examples of several lysosomes visiting the same IP3R punctum at different times (Figure S3B; Video S7), and of a mobile IP3R punctum pausing when it collided with an almost immobile lysosome (Figure S4; Video S8).

Because it was difficult to quantify the dynamic interactions between ER/IP3Rs and lysosomes, we used in situ proximity ligation assays (PLA) to report whether an ER (VAP-A) and a lysosomal protein (Rab7) were located within ~40 nm of each other (Figure 5A). PLA detected many spots in HEK cells, indicative of VAP-A/Rab7 proximity, but not when either primary antibody was omitted, confirming the specificity of the PLA (Figures 5B and 5C) or their dynamic interactions (Figures S6A–S6C). We obtained similar results in EGFP-IP3R1-HeLa cells (Figure 5D), where PLA reported the proximity of both VAP-A and EGFP-IP3R to both Rab7 and another lysosomal protein (LAMP1) (Figures 5E and 5F). Furthermore, EGFP-IP3R puncta and the PLA spots indicative of VAP-A/LAMP1 proximity were significantly colocalized (Manders’s split coefficient, 0.70 ± 0.21, n = 18 cells, Costes’s p value, 100%) (Figure 5D). Hence the MCSs between ER and lysosomes (revealed by the proximity of VAP-A to Rab7 or LAMP1) are populated by IP3R puncta. Collectively, our results suggest that lysosomes preferentially associate with regions of ER where there are IP3R puncta (Figures 4, 5, and S2–S4).

We considered whether IP3Rs might contribute to formation of ER-lysosome MCSs, but two lines of evidence suggest this is unlikely. First, in HEK cells with and without IP3R the association of lysosomes with ER was indistinguishable whether determined by PLA (Figures 5B and 5C) or their dynamic interactions (Figures 6A–6C; Videos S9 and S10). Second, we reported previously that the [Ca2+]i increase evoked by inhibiting SERCA was exaggerated by bafilomycin A1. We speculated that these signals might be due to Ca2+ leaking from the ER through translocons, IP3Rs, or other unidentified channels (López Sanjurjo et al., 2013). However, bafilomycin A1 potentiated the increase in [Ca2+]i evoked by addition of thapsigargin in Ca2+-free HBS to a similar extent in HEK cells with and without IP3Rs (Figures 6D–6F). Similar results were obtained with HAP1 cells in which all endogenous IP3R genes were disrupted (Figure S5). These results indicate that IP3Rs mediate transfer of Ca2+ from ER to lysosomes in cells stimulated with CCh, but additional unidentified Ca2+ leak channels in the ER can deliver Ca2+ to lysosomes in unstimulated cells. We conclude that IP3Rs associate with stable ER-lysosome MCSs, but they are not required for their assembly.

MCSs between ER and lysosomes are probably stabilized by tether proteins that may include VAP or protrudin (in ER), and STARD3, ORP1L, Rab7, TPC2, or phosphatidylinositol 3-phosphate (in lysosomes) (Kilpatrick et al., 2017; Phillips and Voeltz, 2016; Raiborg et al., 2015; Rocha et al., 2009). We manipulated ORP1L, which facilitates ER-lysosome contacts when cholesterol levels are low (Rocha et al., 2009), to assess whether it contributes to assembling the MCS where the Ca2+ exchanges occur. Expression of either ORP1L or forms expected to stabilize (∆ORD) or destabilize (∆ORDPHDPhD) ER-lysosome MCSs (Figure S6A) did not significantly affect potentiation of CCh-evoked Ca2+ signals by CCA (Figures S6B and S6C). However, expression of each ORP1L protein reduced the amplitude of the CCh-evoked Ca2+ signal (Figure S6B), but because this effect had no clear relationship to the expected effects of ORP1L proteins on MCS, we have not explored it further.

**A Sustained Increase in Lysosomal pH Disrupts Lysosome Distribution and Ca2+ Handling**

The Ca2+ uptake mechanism in mammalian lysosomes is unknown. It has been suggested to involve a low-affinity Ca2+-H+ exchanger (CAX), but there is no known CAX in mammalian cells (Melchionda et al., 2016; Morgan et al., 2011).

We used the ratiometric pH indicator, dextran-conjugated fluorescein, to measure lysosomal pH (Canton and Grinstein, 2013; Johnson et al., 2016). CCA (1 μM) caused a slow increase in lysosomal pH that reached a stable value after about 40 min (Figures 7A and 7B). Similar results were obtained when Lyso-Tracker Red was used to report lysosomal pH (Figure 7C). In parallel analyses, we determined the effects of CCA on the Ca2+ signals evoked by CCh. As expected, prolonged incubation with CCA exaggerated the CCh-evoked increase in [Ca2+]i (Figure 7D), consistent with normal lysosomes sequestering Ca2+ released through IP3Rs (Figure 1). However, the effect of CCA on CCh-evoked Ca2+ signals was much slower to develop than its effect on lysosomal pH. There was no evident effect of CCA on Ca2+ signals within 40 min, and a statistically significant potentiation of CCh-evoked Ca2+ signals required a 60-min
Figure 5. PLA Analyses Show IP₃Rs at ER-Lysosome MCSs

(A) PLA uses primary antibodies that recognize proteins in ER or lysosome (LY) membranes. Complementary oligonucleotides conjugated to secondary antibodies hybridize only if they are close to each other. Ligation of the hybridized strands then allows rolling circle amplification (RCA) of the oligonucleotide and incorporation of the red fluorescent probe.

(B) Images of HEK cells with (WT) and without (KO) IP₃Rs from PLA analyses of VAMP-associated protein A (VAP-A) proximity to Rab7. Confocal maximum intensity Z-projections show PLA spots (red) and nuclei (gray). Effects of CcA (1 mM, 1 hr) and omission of either primary antibody (Ab) are shown.

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incubation with CcA (Figure 7D). These results show that Ca\(^{2+}\) sequestration by lysosomes persists after dissipation of the H\(^+\) gradient, suggesting that compromised sequestration of the Ca\(^{2+}\) released through IP\(_3\)Rs may be a secondary consequence of the increase in lysosomal pH.

Lysosomes fuse when their pH increases (Cao et al., 2015) and the least acidic lysosomes are more peripherally located (Johnson et al., 2016). We therefore considered whether the delayed effects of CcA on CCh-evoked Ca\(^{2+}\) signals might be caused by redistribution of lysosomes. In most, although not all, HEK cells, prolonged treatment with CcA caused lysosomes to clearly redistribute from perinuclear to peripheral regions (Figures 7E and S7A); the redistribution was evident, but less striking, in the remaining cells. The same treatment caused a small, but statistically insignificant, decrease in the number of small lysosomes and a significant increase in the number of large ones (Figures 7F and S7B). These changes, suggesting fusion or ineffective fission of lysosomes, are similar to those evoked by knockdown of the core subunit of the V-ATPase (Figure 1F). Hence, prolonged dissipation of the lysosomal H\(^+\) gradient causes lysosomes to enlarge and accumulate peripherally, and might thereby disrupt their interactions with IP\(_3\)Rs and ER. Quantification of the distance between each lysosome and the nearest IP\(_3\)R punctum confirmed that treatment with CcA increased the separation of lysosomes from native IP\(_3\)R (Figures 7G and 7H). PLA analyses independently verified that ER-lysosome MCSs, determined by the proximity of Rab7 to VAP-A or EGFP-IP\(_3\)R\(_1\), were disrupted by CcA (Figures S5B, 5C, 5E, and 5F). Peripheral, less acidic lysosomes express less Rab7 (Johnson et al., 2016), which could perturb our PLA analyses with CcA. We therefore reassessed the effects of CcA using PLA with a LAMP1 antibody to identify lysosomes. The results confirm that treatment with CcA reduces the number of ER-lysosome contacts (Figure 5F).

DISCUSSION

Selective Delivery of Ca\(^{2+}\) to Lysosomes from ER Ca\(^{2+}\) Channels

Dissipating the lysosomal pH gradient using inhibitors of the V-ATPase (López Sanjurjo et al., 2013, 2014) or knockdown of one of its core subunits exaggerates the cytosolic Ca\(^{2+}\) signals evoked by IP\(_3\)Rs, but not those evoked by SOCE (Figures 1 and S1). The effect, which is due to attenuated Ca\(^{2+}\) removal from cytosol rather than enhanced release from ER (López Sanjurjo et al., 2013), indicates that lysosomes selectively sequester Ca\(^{2+}\) released from the ER (Figure 1I). Others have used a Ca\(^{2+}\) sensor targeted to lysosomal membranes (LAMP1-yellow cam-eleon3.6) to detect Ca\(^{2+}\) signals evoked by histamine in HELa cells (McCue et al., 2013), but this sensor with its high affinity for Ca\(^{2+}\) (K\(_{Ca}^0 = 250\) nM) (Nagai et al., 2004) cannot distinguish local from global [Ca\(^{2+}\)] increases. To detect local increases in [Ca\(^{2+}\)]\(_o\), we targeted a low-affinity sensor to lysosome membranes (Ly-GG, K\(_{Ca}^0 = 1.2\) μM) and tracked the responses of individual lysosomes (Video S1). Our results show that IP\(_3\)R subtypes, but not SOCE, deliver Ca\(^{2+}\) to about 60% of lysosomes (Figure 2).

A similar situation prevails for mitochondria, where release of Ca\(^{2+}\) from IP\(_3\)R adjacent to mitochondria generates a high local [Ca\(^{2+}\)]\(_o\), sufficient to allow Ca\(^{2+}\) uptake through the low-affinity mitochondrial uniporter complex (MCU) (Rizzuto et al., 2012). For mitochondria, IP\(_3\)R3 may, at least in some cells, selectively deliver Ca\(^{2+}\) to mitochondria (Cárdenas et al., 2010; Giorgi et al., 2018; Mendes et al., 2005). By contrast, our results show that all three IP\(_3\)R subtypes can deliver Ca\(^{2+}\) to lysosomes (Figure 3). We suggest that for both lysosomes and mitochondria, the ER through its SERCA and IP\(_3\)R provides a route through which Ca\(^{2+}\) can be accumulated from the low [Ca\(^{2+}\)]\(_o\), of resting cells by a high-affinity uptake system (SERCA) and then delivered locally through large-conductance channels (IP\(_3\)R and perhaps others) at a high local concentration to a low-affinity uptake system (MCU or lysosomes) (Figure 7I). The ER, with its high-affinity SERCA and large-conductance Ca\(^{2+}\) channels, behaves like a compressor or piston linking a low [Ca\(^{2+}\)]\(_o\) to the low-affinity uptake systems of organelles (Figure 7I).

IP\(_3\)R subtypes provide a link between extracellular stimuli and delivery of Ca\(^{2+}\) to lysosomes (Figures 1, 2, 3, and S1), but additional unidentified ER Ca\(^{2+}\) channels can deliver Ca\(^{2+}\) to lysosomes in unstimulated cells (Figures 6D–6F and S5). We propose, in keeping with a recent report (Garrity et al., 2016), that microdomains of high local [Ca\(^{2+}\)]\(_o\) presented to lysosomes by IP\(_3\)R may facilitate lysosomal Ca\(^{2+}\) uptake (Figure 7I), and thereby link signaling through PLC to lysosome behavior.

IP\(_3\)R Clusters at ER-Lysosome Contacts Facilitate Ca\(^{2+}\) Transfer

MCS, where membranes of the ER and another organelle are held in close apposition by scaffold proteins, facilitate exchange of materials, including Ca\(^{2+}\), between organelles (Figure 7I) (Phillips and Voeltz, 2016). Within the endosomal pathway, MCSs with the ER become more abundant as endosomes mature and acidify, such that most late endosomes form MCSs with the ER (Friedman et al., 2013) (Figure 4A; Videos S2 and S3). Several integral membrane components of the ER (e.g., VAP,
Figure 6. IP$_3$Rs Are Not Required for ER-Lysosome Contacts

(A) TIRFM images of HEK cells with (WT) and without IP$_3$Rs (KO), expressing LAMP1-mCherry and EGFP-ER.
(B) Enlargements of boxed region in (A) show associations of lysosomes and ER.
(C) Time series (3-s intervals) of boxed regions in (B) show dynamics of ER-lysosome interactions (from Videos S9 and S10).
(D) HEK cells were treated with bafilomycin A1 (Baf A1, 1 μM, 1 hr) in HBS before addition of BAPTA (2.5 mM) and then thapsigargin (1 μM). Mean ± SD from three wells in one experiment.
(E and F) Summary results show peak thapsigargin-evoked Ca$^{2+}$ release in WT (E) and KO cells (F) as paired values (each with three determinations) and mean (n = 4, line). *p < 0.05, paired Student’s t test.

See also Figures S5 and S6 and Videos S9 and S10.
protrudin) and endosomes (e.g., STARD3, ORP1L, Rab7, phosphatidylinositol 3-phosphate) are implicated in the formation of MCSs between the ER and late endosomes (Alpy et al., 2013; Eden, 2016; Hong et al., 2017; Phillips and Voeltz, 2016; Raiborg et al., 2015), and both Ca²⁺ signals within MCSs and functional TPC1 may be required for their maintenance (Kilpatrick et al., 2017). The components of lysosome-ER MCSs are less defined, although it has been suggested that TPC2 may fulfill a similar role here to that suggested for TPC1 at endosome-ER MCSs (Kilpatrick et al., 2017). Hence, although it is widely supposed that MCSs mediate exchange of, for example, Ca²⁺ and cholesterol, between ER and lysosomes, the composition of these MCSs is unclear. Our analyses suggest that ORP1L is unlikely to be an essential component of the MCSs wherein Ca²⁺ is delivered from the ER to lysosomes (Figure S6).

We confirmed that most lysosomes maintain contact with the ER, despite movements of both organelles (Figure 4A; Videos S2 and S3) (López Sanjurjo et al., 2013). The association reflects close apposition of the ER and lysosome membranes (Figure 5). The most persistent associations between lysosomes and the ER coincide with regions that are populated by clusters of native IP₃Rs, and the affiliation of IP₃Rs with lysosomes occurs at both moving and immotile contacts (Figures 4, 5D–5F, and S2–S4; Videos S4, S5, S6, S7, and S8). Although IP₃R₃s populate the...
ER-lysosome MCSs, our results suggest they do not contribute to assembly of the MCSs. Sequestration by lysosomes of Ca\textsuperscript{2+} released from the ER through leak channels is similar in cells with and without IP\textsubscript{3}R puncta (Figures 4C−4E and S2−S4; Videos S4, S5, S6, S7, and S8), is similar in cells with and without IP\textsubscript{3}R puncta (Figures 4A−4C; Videos S9 and S10), and the proximity of ER (VAP-A) and a lysosome protein (Rab7) determined by PLA is indistinguishable in cells with and without IP\textsubscript{3}R (Figures 5B and 5C). We conclude that IP\textsubscript{3}R puncta associate with stable ER-lysosome MCSs, but IP\textsubscript{3}R are not required for assembly of these contacts.

How Do Lysosomes Accumulate Ca\textsuperscript{2+}?

Ca\textsuperscript{2+} uptake by mammalian lysosomes has been suggested to involve a low-affinity CAX, consistent with evidence that dissipating the lysosomal pH gradient increases [Ca\textsuperscript{2+}].\textsubscript{L} (Lloyd-Evans et al., 2008) and decreases lysosomal free [Ca\textsuperscript{2+}] (Christensen et al., 2002), with Ca\textsuperscript{2+} uptake causing an increase in lysosomal pH (Hilden and Madias, 1989; López Sanjurjo et al., 2013; Morgan and Galione, 2007) and with heterologous expression of Xenopus CAX in mammalian cells attenuating CCh-evoked Ca\textsuperscript{2+} signals (Melchionda et al., 2016). However, there is no known CAX in mammalian cells (Melchionda et al., 2016; Morgan et al., 2011).

Our results show that, although dissipating the lysosomal pH gradient inhibits Ca\textsuperscript{2+} uptake by lysosomes, there is a temporal mismatch between the effects of CcA on lysosomal pH and attenuated Ca\textsuperscript{2+} uptake, with the latter developing much more slowly (Figures 7A−7D). A recent study, in which a low-affinity Ca\textsuperscript{2+} sensor tethered to TRPML1 was used to report refilling of lysosomes, likewise concluded that treatments with bafilomycin A\textsubscript{1}, or CcA that abolished the lysosomal pH gradient did not prevent refilling of lysosomes with Ca\textsuperscript{2+} (Garrity et al., 2016). Hence, and consistent with the apparent absence of CAX from mammalian cells, lysosomes can, at least acutely, accumulate Ca\textsuperscript{2+} in the absence of a pH gradient. Why then does sustained inhibition of the V-ATPase prevent lysosomes from sequestering Ca\textsuperscript{2+} released from the ER (Figures 1, 3, and S1)?

We suggest that this inhibition results from disruption of the contacts between ER and lysosomes. This is consistent with evidence that vacuolin, which fuses lysosomes (Huynh and Andrews, 2005), also prevents lysosomes from sequestering Ca\textsuperscript{2+} released from the ER (López Sanjurjo et al., 2013). Sustained inhibition of the V-ATPase causes the fraction of the cell occupied by lysosomes to decrease by ∼20% (Abu-Remaileh et al., 2017), enlargement of lysosomes (Figures 1F, 7F, and S7), redistribution of lysosomes from perinuclear to peripheral regions (Figures 7E and S7), and disruption of both ER-lysosome MCSs (Figures 5B, 5C, 5E, and 5F) and the association of IP\textsubscript{3}R with lysosomes (Figures 5D−5F, 7G, and 7H). These observations are consistent with results showing that lysosomal pH and/or local Ca\textsuperscript{2+} signals regulate fusion and/or fission of lysosomes (Baars et al., 2007; Cao et al., 2017; Coen et al., 2012; Kilpatrick et al., 2017; Ruas et al., 2010) and their subcellular distribution (Johnson et al., 2016). We suggest that the inability of lysosomes to sequester Ca\textsuperscript{2+} released from ER after sustained inhibition of the V-ATPase results from a disruption of the MCSs where Ca\textsuperscript{2+} transfer occurs between lysosomes and ER (Figure 7J). Our evidence that CcA increases the separation of lysosomes and native IP\textsubscript{3}R supports this suggestion (Figures 5D−5F, 7G, and 7H). We have not determined whether all Ca\textsuperscript{2+} uptake by lysosomes occurs at these MCSs, but others have suggested that IP\textsubscript{3}R-mediated transfer from the ER is the only means by which lysosomes sequester Ca\textsuperscript{2+} (Garrity et al., 2016). We suggest that MCSs between ER and lysosomes are populated by clusters of IP\textsubscript{3}R, poised to deliver Ca\textsuperscript{2+} at high local concentrations to a low-affinity, but as yet unidentified, lysosomal Ca\textsuperscript{2+} uptake mechanism (Figures 7I). Because the distribution of lysosomes and their association with ER are regulated by amino acids (Hong et al., 2017), it is likely that dynamic regulation of ER-lysosome MCSs also regulates lysosomal Ca\textsuperscript{2+} uptake.

STAR METHODS

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Supplemental Information

Supplemental Information includes seven figures, one table, and ten videos and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.11.064.

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Author Contributions

P.A. and N.B.T. performed most experiments. S.M. characterized HAP1 cells. D.L.P. contributed to development of G-GECO sensors. C.W.T. supervised the
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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Donkey anti-rabbit IgG-HRP (WB, 1:5000) | Santa Cruz Biotechnology Inc, Dallas, TX | Cat# sc-2313; RRID: AB_641181 |
| Goat anti-mouse IgG-HRP (WB, 1:5000) | Santa Cruz Biotechnology | Cat# sc-2005; RRID: AB_631736 |
| Rabbit anti-IP3R1 (WB, 1:1000) | Cell Signaling Technology, Boston, MA | Cat# 3763; RRID: AB_2129958 |
| Rabbit anti-IP3R2 (WB, 1:1000) | Custom-made to peptide (GFLGSTOPHENHHMPPH) by Pocono Rabbit Farm and Laboratory, Inc, Canadensis, PA. (Mataragka and Taylor, 2018) | n/a |
| Mouse anti-IP3R3 (WB, 1:1000) | BD Biosciences, Wokingham, UK | Cat# 610312; RRID: AB_397704 |
| Mouse anti-β-actin (WB, 1:1000) | Cell Signaling Technology | Cat# 3700; RRID: AB_2242334 |
| Rabbit (monoclonal) anti-ORP1 (WB, 1:1000) | Abcam, Cambridge | Cat# ab131165; RRID: AB_11155305 |
| Mouse anti-VAP-A (PLA, 1:100) | Santa Cruz Biotechnology | Cat# sc-293278 |
| Rabbit anti-Rab7 (PLA, 1:100) | Cell Signaling Technology | Cat# 9367; RRID: AB_1904103 |
| Mouse anti-GFP (PLA, 1:500) | ThermoFisher, Paisley, UK | Cat# A-11120; RRID: AB_221568 |
| Rabbit (monoclonal) anti-LAMP1 (PLA, 1:200) | Cell Signaling Technology | Cat# 9091; RRID: AB_2687579 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| ATP disodium salt | Sigma-Aldrich | Cat# A9187 |
| Alexa Fluor™ 488-dextran conjugate (10,000 MW) | ThermoFisher | Cat# D22910 |
| Bafilomycin A1 (Baf A1) | Fluorochem, Hadfield, UK | Cat# M01404 |
| Bafilomycin A1 (Baf A1) | Alfa Aeser via ThermoFisher | Cat# JS1835 |
| BAPTA | Molekula, Dorset, UK | Cat# 20358510 |
| Bovine serum albumin (BSA) | Europa Bioproducts, Cambridge, UK | Cat# EQBAH64 |
| Caged cell-permeant IP3 (ci-IP3 PM) | SiChem, Bremen, Germany | Cat# cag-iso-2-145-100 |
| Carbachol (carbamoylcholine chloride, CCh) | Sigma-Aldrich, Gillingham, UK | Cat# Y0000113 |
| cOmplete EDTA-free protease inhibitor cocktail | Sigma-Aldrich | Cat# 11873580001 |
| Concanamycin A (CcA) | Insight Biotechnology, Middlesex, UK | Cat# sc-202111A |
| Cyclopiazonic acid (CPA) | Bio-Technne, Minneapolis, MN | Cat# 1235 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | Cat# D2650 |
| DMEM/F-12, GlutaMAX medium | ThermoFisher | Cat# 31331028 |
| ECL Prime chemiluminescence detection reagent | GE Healthcare, Little Chalfont, UK | Cat# RP22232 |
| Fetal bovine serum (FBS) | Sigma-Aldrich | Cat# F7524, batch 094M3341 |
| Fibronectin (human) | Merck Millipore, Watford, UK | Cat# FC010 |
| Fluorescein-conjugated dextran (10,000 MW, Fluoro-Emerald) | ThermoFisher | Cat# D1820 |
| Fluo 8-AM | Stratech Scientific, Suffolk, UK | Cat# 21080-AAT |
| HEPES | Merck Millipore | Cat# 391338 |
| Histamine dihydrochloride | Sigma-Aldrich | Cat# H7250 |
| Inositol 1,4,5-trisphosphate (IP3) | Enzo, Exeter, UK | Cat# BML-CA430-0001 |
| Ionomycin | Apollo Scientific, Stockport, UK | Cat# 56092-81-0 |
| Isocove’s Modified Dulbecco’s Medium (IMDM) with GlutaMAX | ThermoFisher | Cat# 12440-05 |
| LysoTracker Red DND-99 | ThermoFisher | Cat# L7528 |
| Mag-fluor 4-AM | Cambridge Bioscience, Cambridge, UK | Cat# M-14206 |

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**Continued**

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| NucBlue Live Ready Probe | ThermoFisher | Cat# R37606 |
| PIPES | Sigma-Aldrich | Cat# P1851 |
| Pluronic F-127 | Sigma-Aldrich | Cat# P2443 |
| Poly-L-lysine | Sigma-Aldrich | Cat# P8920 |
| Restriction enzyme: BamHI | Fermentas via ThermoFisher | Cat# FD0054 |
| Restriction enzyme: EcoRI | Fermentas via ThermoFisher | Cat# FD0274 |
| Restriction enzyme: HindIII | Fermentas via ThermoFisher | Cat# FD0504 |
| Saponin | Sigma-Aldrich | Cat# S4521 |
| siPORT NeoFX transfection reagent | ThermoFisher | Cat# AM4511 |
| T4 DNA ligase | ThermoFisher | Cat# M0202S |
| Thapsigargin | Bio-Techn, Minneapolis, MN | Cat# 1138 |
| TransIT-LT transfection reagent | GeneFlow, Lichfield, UK | Cat# E7-0002 |
| Tris base | ThermoFisher | Cat# BP152-1 |
| Triton X-100 | Sigma-Aldrich | Cat# T8787 |
| TrypLE Express | ThermoFisher | Cat# 12605010 |
| Tween-20 | Sigma-Aldrich | Cat# TS927 |
| U73122 | Bio-Techn | Cat# 1268/10 |
| U73343 | Bio-Techn | Cat# 4133/10 |

**Critical Commercial Assays**

| REAGENT | SOURCE | IDENTIFIER |
|---------|--------|------------|
| DC™ protein assay kit II | BioRad, Watford, UK | Cat# 5000112 |
| Duolink in situ Red Starter kit mouse/rabbit | Sigma-Aldrich | Cat# DUO92101-1KT |
| Duolink in situ PLA probe: anti-rabbit PLUS, affinity-purified donkey anti-rabbit IgG | Sigma-Aldrich | Cat# DUO92002-100RXN |
| Duolink in situ PLA probe: anti-mouse MINUS, affinity-purified donkey anti-mouse IgG | Sigma-Aldrich | Cat# DUO92004-100RXN |
| Duolink in situ detection reagents Red | Sigma-Aldrich | Cat# DUO92008-100RXN |
| Duolink in situ mounting medium | Sigma-Aldrich | Cat# DUO82040 |
| Duolink in situ wash buffer fluorescence | Sigma-Aldrich | Cat# DUO82049 |
| FastLane cell cDNA kit | QIAGEN, Crawley, UK | Cat# 215011 |
| Plasmid maxi kit | QIAGEN | Cat# 12165 |
| QIAquick gel extraction kit | QIAGEN | Cat# 28706 |
| QIaprep spin miniprep kit | QIAGEN | Cat# 27104 |
| QIaprep endoFree plasmid maxi kit | QIAGEN | Cat# 12362 |
| Rotor-Gene SYBR Green PCR kit | QIAGEN | Cat# 204074 |

**Experimental Models: Cell Lines**

| REAGENT | SOURCE | IDENTIFIER |
|---------|--------|------------|
| EGFP-IP<sub>3</sub>R1-HeLa cells | (Thillaiappan et al., 2017) | n/a |
| HAP1 cells without IP<sub>3</sub>Rs | Horizon Discovery, Cambridge, UK | n/a |
| HEK cells | Dr David Yule, University of Rochester, NY (parental cell line from which HEK cells lacking IP<sub>3</sub>R subtypes were generated); (Alzayady et al., 2016) | n/a |
| HEK cells expressing single native IP<sub>3</sub>R subtypes | Kerastat, Boston, MA (cell lines generated by Dr Yule); (Alzayady et al., 2016) | Cat# EUR031, EUR032, EUR033, EUR034, EUR035 and EUR036 |

**Oligonucleotides**

| REAGENT | SOURCE | IDENTIFIER |
|---------|--------|------------|
| Silencer<sup>TM</sup> siRNA against human ATP6V0C | ThermoFisher | Cat# 4390824 |
| Silencer<sup>TM</sup> Select siRNA against human ORP1L (also known as OSBPL1A) | ThermoFisher | Cat# s41681 and s41682 |

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## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Cell Culture and Transfection

The methods used to establish EGFP-IP3R1-HeLa cells, in which all endogenous IP3R1 are N-terminally tagged with monomeric EGFP have been fully described (Thillaiappan et al., 2017). In brief, we used gene-editing with transcription activator-like effector nucleases (TALENs) to modify both copies of the IP3R1 gene. Sequencing alongside functional and optical microscopy analyses, confirmed both the selectivity of the editing and that the edited IP3Rs form functional Ca2+ release channels (Thillaiappan et al., 2017). HEK cells, in which CRISPR/Cas9 was used to delete one or more IP3R subtypes, were generated by Dr David Yule’s laboratory (University of Rochester, NY) (Alzayady et al., 2016) and supplied by Kerafast. HAP1 cells, genetically engineered using CRISPR/Cas9 to disrupt genes for all three IP3R subtypes, were developed in collaboration with Horizon Discovery (Cambridge, UK). Short tandem repeat profiling was used to authenticate the HeLa cells used (Eurofins, Germany) and the HEK cells lacking

### REAGENT OR RESOURCE SOURCE IDENTIFIER

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Silencer negative control No.1 siRNA | ThermoFisher | Cat# AM4611 |
| Primers for sequencing and construction of plasmids, see Table S1. | ThermoFisher | n/a |
| QuantiTect QPCR primer for human GAPDH | QIAGEN | Cat# QT00079247 |
| QuantiTect QPCR primer for human ATP6V0C | QIAGEN | Cat# QT00220738 |

### Recombinant DNA

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pcDNA3.1(+) plasmid | ThermoFisher | Cat# V790-20 |
| G-GECO1.2 (CyGG) | Addgene (Zhao et al., 2011) | Cat# 32446 |
| LAMP1-mCherry | (López Sanjurjo et al., 2013) | n/a |
| LAMP1-G-GECO1.2 (Ly-GG) in pcDNA3.1(+) | This study | n/a |
| mTurquoise-LAMP1 (we note that despite the nomenclature, the LAMP1 is tagged at its C terminus with mTurquoise in this construct) | Addgene, deposited by Michael Davidson, Florida State University | Cat# 55568 |
| mCherry-ER | Addgene, deposited by Michael Davidson, Florida State University | Cat# 55041 |
| TPC2-mRFP | (Brailoiu et al., 2009) | n/a |
| TPC2-GFP | (Brailoiu et al., 2009) | n/a |
| mRFP-ORP1L and variants, see Figure S6: | Dr J Neefjes (University of Leiden Medical Center, the Netherlands) | n/a |
| ΔORD | (Rocha et al., 2009) | n/a |
| mRFP-ΔORDPHDPHD | n/a |
| mRFP-ΔORD | n/a |

### Software and Algorithms

| SOFTWARE AND ALGORITHMS | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| BioEdit, version 7.0.5 | Ibis Therapeutics, North Carolina State University, NC | http://www.mbio.ncsu.edu/BioEdit |
| CellProfiler, version 2.1 | n/a | http://cellprofiler.org |
| Clustal Omega | n/a | https://www.ebi.ac.uk/Tools/msa/clustalo |
| Fiji/ImageJ | (Schindelin et al., 2012) | http://fiji.sc/ |
| GeneTools, version 4 | Syngene, Cambridge, UK | https://www.syngene.com/ |
| MetaMorph Microscopy Automation and Image Analysis | Molecular Devices, San Jose, CA | https://www.moleculardevices.com/ |
| Prism 5, version 5 | GraphPad, La Jolla | https://www.graphpad.com/ |
| SoftMax Pro, version 7 | Molecular Devices, San Jose, CA | https://www.moleculardevices.com/ |
all three IP_{3}R subtypes (Public Health England). We have not confirmed the authenticity of the HAP1 cell lines. Regular screening throughout the study established that all cell lines were free of mycoplasma.

HeLa and HEK293 cell lines were cultured in Dulbecco’s Modified Eagles Medium (DMEM)/F-12 with GlutaMAX supplemented with fetal bovine serum (FBS, 10%). HAP1 cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) with 10% FBS. All cells were maintained at 37°C in humidified air with 5% CO_{2}, and passaged every 3-4 days using TrypLE Express.

For imaging, cells were grown on 35-mm glass-bottomed dishes (#P35G-1.0-14-C, MatTek Corporation, Ashland, MA, USA; or D35-14-1-N, IBL Baustoff-Labor, Austria) coated with human fibronectin (10 μg/ml). Cells were transfected with plasmids encoding Ca^{2+} indicators (Ly-GG or Cy-GG) or targeted proteins according to the manufacturer’s instructions using TransIT-LT1 reagent (1 μg DNA/2.5 μl reagent). For siRNA transfections, cells were plated in clear-bottomed 96-well plates (Greiner Bio-One, Stonehouse, UK) coated with poly-L-lysine (0.01% w/v). After 24 hr, cells were transfected with Silencer<sup>TM</sup> siRNA (40 nM) directed against ATP6V0C or a non-silencing control siRNA using siPORT NeoFX transfection reagent (220 ng siRNA/μl reagent). Experiments were performed 48-72 hr after transfection. The same methods were used for transfection with siRNA directed against human ORP1L (Alpy et al., 2013) (Figure S6).

**METHOD DETAILS**

**Plasmids**

The genetically-encoded, low-affinity Ca^{2+} sensor G-GECO1.2 (equilibrium dissociation constant for Ca^{2+}, K_{D}Ca = 1.2 μM) (Zhao et al., 2011) was used to record [Ca^{2+}]_{c}. The initial templates for cloning of a low-affinity Ca^{2+} sensor targeted to the cytosolic surface of the lysosomal membrane (Ly-GG) were LAMP1-mCherry and cytosolic G-GECO1.2. A HindIII recognition site was inserted at the 5’ end of LAMP1-mCherry using primer LAMP1F (Table S1). LAMP1-mCherry has a pre-existing BamHI site. Primers LAMP1F and LAMP1R were used to amplify the LAMP1 sequence from LAMP1-mCherry using PCR. The LAMP1 PCR product was digested with HindIII and BamHI. A BamHI site was introduced in-frame with the 5’ end of G-GECO1.2 by PCR using primer G-GECO1.2F, and an EcoRI site was introduced at the 3’ end of G-GECO1.2 using primer G-GECO1.2R. The product was then digested with BamHI and EcoRI. The LAMP1-G-GECO1.2 construct was assembled in pcDNA3.1(+) expression vector. pcDNA3(+) was digested with HindIII and EcoRI overnight to create sticky ends suitable for ligation with the LAMP1 and G-GECO1.2 fragments. The digested LAMP1, G-GECO1.2 and pcDNA3.1(+) were ligated using T4 DNA ligase according to the manufacturer’s protocol. The complete coding sequence of LAMP1-G-GECO1.2 was verified using the following primers: LAMP1SeqF1, LAMP1SeqF2, primer G-GECO1.2SeqM and GGECO1.2SeqE (Table S1). Sequencing data were analyzed using BioEdit software, and alignments were carried out using Clustal Omega. The cytosolic and lysosome-targeted G-GECO1.2 s are described as Cy-GG and Ly-GG in the text. Plasmids encoding TPC2-mRFP and TPC2-GFP (Brailoiu et al., 2009), LAMP1-GFP (López Sanjurjo et al., 2013), mTurquoise-LAMP1, mCherry-ER and LAMP1-mCherry (López Sanjurjo et al., 2013) have been described. Plasmids encoding ORP1L and its variants (Rocha et al., 2009) (Figure S6) were provided by Dr J Neefjes (University of Leiden Medical Center, the Netherlands).

**Measurements of [Ca^{2+}]_{c} in Cell Populations**

Confluent monolayers of cells grown in a 96-well plate (Greiner Bio-One, Storehouse, UK) were loaded with fluo 8 by incubation for 1 hr at 20 °C in HEPES-buffered saline (HBS, 100 μl) containing fluo 8-AM (2 μM) and 0.02% Pluronic F-127. Cells were then washed and incubated in HBS for 1 h at 20 °C to allow de-esterification of fluo 8-AM. HBS had the following composition: 135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl_{2}, 1.5 mM CaCl_{2}, 11.5 mM D-glucose, 11.6 mM HEPES, pH 7.3. CaCl_{2} was omitted from nominally Ca^{2+}-free HBS. In some experiments, BAPTA (final concentration 2.5 mM) was added to HBS immediately before stimulation to reduce the free [Ca^{2+}]_{c} of the HBS to < 20 nM. Fluorescence was recorded using a FlexStation III fluorescence plate-reader (Molecular Devices, Sunnyvale, CA, USA) (López Sanjurjo et al., 2013). Fluorescence was recorded at 1.44-s intervals, with excitation at 485 nm and emission at 525 nm. Data were collected and analyzed using SoftMax Pro software. Maximal (Fmax) and minimal (Fmin) fluorescence values were determined from parallel wells after addition of Triton X-100 (0.1%) to lyse cells in the presence of either 10 mM CaCl_{2} (Fmax) or 10 mM BAPTA (Fmin). Fluorescence values (F) were calibrated to [Ca^{2+}]_{c} using a KD = 389 nM from:

\[
[Ca^{2+}]_c = K_D \times \frac{(F - F_{min})}{(F_{max} - F)}
\]

IP_{3}-evoked Ca^{2+} release from saponin-permeabilized HAP1 cells was measured in cytosol-like medium (CLM) using a low-affinity Ca^{2+} indicator (Mag-fluo 4) trapped within the ER, as previously described for other cell types (Tovey et al., 2006). Briefly, cells were loaded with the indicator by incubation with 20 μM Mag-fluo 4-AM in HBS containing BSA (1 mg/ml) and pluronic acid (0.02%, v/v). After 1 hr at 20 °C, cells were resuspended in Ca^{2+}-free CLM, which had the following composition: 140 mM KCl, 20 mM NaCl, 1 mM EGTA, 2 mM MgCl_{2} and 20 mM PIPES, pH 7.0. The plasma membrane was then permeabilized by incubation with saponin (10 μg/ml, 2-3 min, 37 °C). Cells were recovered (600 g, 2 min), re-suspended (~10^6 cells/ml) in Mg^{2+}-free CLM, distributed (45 μl/well) into black half-area 96-well plates and centrifuged (300 g, 2 min). Mag-fluo 4 fluorescence (excitation at 485 nm, emission at 525 nm) was recorded at 1.44-s intervals at 20 °C using a FlexStation III fluorescence plate-reader. Addition of MgATP...
(1.5 mM) allowed Ca\(^{2+}\) uptake into the intracellular stores. When steady-state Ca\(^{2+}\) loading was achieved (~2 min), IP\(_3\) was added with cyclopiazonic acid (CPA, 10 \(\mu\)M) to inhibit further Ca\(^{2+}\) uptake. IP\(_3\)-evoked Ca\(^{2+}\) release is reported as the fractional decrease in the ATP-dependent Mag-fluo 4 fluorescence.

**Fluorescence Microscopy**

Fluorescence microscopy used an inverted Olympus IX83 microscope equipped with a 100x objective (numerical aperture, NA, 1.49), a multi-line laser bank (405, 425, 488, 561 and 647 nm) and an iLas\(_2\) targeted laser illumination system (Cairn, Faversham, UK). Excitation light was transmitted through either a quad dichroic beam-splitter (TRF89002-QUAD) or a dichroic mirror (for 425 nm; ZT442RDC-U2F, Chroma, Germany). Emitted light was passed through appropriate filters (Cairn Optospin; peak-bandwidth: 450/50, 480/40, 525/50, 630/75 and 700/75 nm) and detected with either an iXon Ultra 897 electron multiplied charge-coupled device (EMCCD) camera (512 \(\times\) 512 pixels, Andor, Belfast, Northern Ireland) or (for Figures 4B–4E and S2–S4) a Prime 95B Scientific Complementary Metal Oxide Semiconductor (sCMOS) camera (1200 \(\times\) 1200 pixels, Photometrics, Tucson, AZ, USA). For all multi-color imaging, we confirmed that there was no bleedthrough between channels. For TIRFM, the penetration depth was 90-140 nm. The iLas\(_2\) illumination system was used for TIRFM and wide-field imaging. Bright-field images were acquired using a Cairn MonoLED illuminator. All fluorescence images were corrected for background by subtraction of fluorescence collected from a region outside the cell. Image capture and processing used MetaMorph Microscopy Automation and Image Analysis software.

For colocalization analyses, we used either Pearson’s correlation coefficient (\(R_{\text{coloc}}\)) for comparisons of fluorophores in every pixel, or Manders’ split coefficient to identify the fraction of PLA spots that colocalized with EGFP-IP\(_3\)R1 (Figure 5D). We confirmed, using the Costes randomization method with 100 iterations and ignoring pixels in which there was no fluorescence (Costes et al., 2004), that any colocalization was more than expected from randomly distributed fluorophores (ImageJ Colocalization Analysis/Colocalization Test). \(R_{\text{coloc}}\) was calculated from all pixels within the region of interest (ROI) that exceeded a threshold value (ImageJ Colocalization Analysis/Colocalization Threshold): 

\[
R_{\text{coloc}} = \frac{\sum (Ri - Rm)(Gi - Gm)}{\sqrt{\sum (Ri - Rm)^2 \sum (Gi - Gm)^2}}
\]

where, \(Gi\) and \(Ri\) are the intensities of individual green and red pixels respectively, and \(Gm\) and \(Rm\) are the mean intensities of green and red pixels.

To measure center-center distances between each lysosome (mTurquoise-LAMP1) and the nearest EGFP-IP\(_3\)R1 punctum (Figures 7G and 7H), images were Gaussian-filtered to remove noise, and then analyzed using the ImageJ Distance Analysis plug-in (DiAna) (Gilles et al., 2017).

**Measurement of Near-Lysosome Ca\(^{2+}\) Signals**

HeLa cells transfected with Ly-GG were washed three times in HBS, and Ly-GG fluorescence (excitation at 488 nm, emission at 525 nm) was imaged using wide-field fluorescence microscopy (1 frame/s) at 20°C. After background correction, the Ly-GG fluorescence associated with single lysosomes was measured using single-particle tracking with the MetaMorph Track Objects plugin (Meijering et al., 2012). A template-match algorithm was used to connect tracks between successive frames. Tracks that terminated before completion of the recording (240 s for cells stimulated with histamine; 1330 s for analyses of SOCE) were excluded from the analysis. In parallel analyses of HeLa cells expressing Cy-GG, ROIs similar in dimensions to tracked lysosomes (radius \(\sim 1.6 \mu\)m) were selected for analysis.

**Photolysis of Caged-IP\(_3\)**

HeLa cells grown on fibronectin-coated glass-bottomed dishes were first transfected with Ly-GG or Cy-GG (1 \(\mu\)g/\(\mu\)l, 24 hr), then loaded with ci-IP3/PM (1 \(\mu\)M, 50 min) (Dakin and Li, 2007). After washing and incubation in HBS for a further 45 min, cells were imaged (20°C) using an inverted Olympus IX83 microscope equipped with a 100x objective. Ly-GG fluorescence was recorded in widefield (488 nm excitation, 525/50 nm emission). Cells were imaged for 50 s before photolysis of ci-IP3 using a SPECTRA X-light engine (Lumencor, 395/20 excitation, exposure time 50 ms/frame for 10 frames). Images were acquired at 50-ms intervals with an iXon Ultra 897 EMCCD camera, corrected for background fluorescence, and analyzed using MetaMorph. Ly-GG was tracked to determine Ca\(^{2+}\) signals around single lysosomes. Photolysis of ci-IP3 releases an active, but more metabolically stable, analog of IP3 (i-IP3, in which the 2- and 3-hydroxyls are linked by an isopropylidene group) (Dakin and Li, 2007).

**Measurement of Lysosomal pH**

The pH within lysosomes was measured from defined ROI within single cells using a dextran-conjugated ratiometric pH indicator, fluorescein-dextran, loaded into lysosomes by endocytosis. Cells were incubated with fluorescein-dextran (10-kDa, 0.2 mg/ml) for 16 hr, followed by a 4-hr chase in DMEM F12 at 37°C. The cells were then washed 3 times with HBS, and imaged immediately with alternating excitation/emission (\(F_{425;}\lambda_{ex} = 425\ nm, \lambda_{em} = 480\ nm, F_{488;}\lambda_{ex} = 488\ nm, \lambda_{em} = 525\ nm\)). Images were collected for 100 ms, with 5 min between each round of data acquisition. After background subtraction, ROIs were drawn around lysosomes
clusters and fluorescence ratios (R, which increases with increased pH) were calculated from F488/F425 at each time. Results are presented as R/R0, where R0 is the fluorescence ratio recorded before stimulation.

For experiments with LysoTracker Red, cells were loaded with 50 nM LysoTracker Red DND-99 for 1 hr, washed 3 times with HBS, and imaged immediately with excitation and emission at 561 nm and 630 nm, respectively.

**Measurement of Lysosome Size**

Lysosome size was measured in HEK cells using either LAMP1-mCherry (Figures 7E and 7F) or endocytosed Alexa Fluor 488-dextran (10,000, MW) (Figure S7) to identify lysosomes. After application of a threshold (ImageJ Threshold), particles were accepted for analysis if they had a circularity value (4π.area/circumference2) of 0.6-1.0. The circularity criterion ensured that only roughly circular particles were selected for analysis (Grossi et al., 2016). Visual inspection of images before and after application of the selection criteria confirmed that most lysosomes were included in the final analysis. We use the Feret diameter to report lysosome size, which is the maximum distance between two points on the perimeter of the particle (ImageJ Analyze Particles) (Ferraro et al., 2014).

**Quantitative PCR**

QPCR was carried out as previously described (Tovey et al., 2008). cDNA was synthesized in a final volume of 20 μl from a lysate prepared from confluent cells in 1 well of a 96-well plate, using a FastLane cell cDNA kit. For QPCR, each reaction included primers for ATP6V0C and, for calibration, primers for a housekeeping gene (glyceraldehyde phosphate dehydrogenase, GAPDH). Each reaction (20 μl) included Rotor-Gene SYBR Green PCR master mix (10 μl), cDNA (5 μl), Quantitect primer assay (2 μl) and RNAase-free water (3 μl). In two negative controls, the primers were omitted during QPCR, or the reverse transcriptase was omitted during cDNA synthesis. For QPCR (Rotor-Gene 6000, Corbett Life Sciences), an initial denaturation at 95 °C for 5 min was followed by 40 cycles of amplification (93 °C for 5 s, 60 °C for 10 s) and then a melting curve (72 °C to 95 °C). Expression of mRNA relative to that for GAPDH was calculated from:

\[
\text{Expression} = \frac{E^{C_{\text{ATP6V0C}}}}{E^{C_{\text{GAPDH}}}}
\]

E is the amplification efficiency, calculated as 10m, where m is the average fluorescence increase for the four cycles after the cycle threshold (Ct) for the indicated PCR product. Results are reported as mean ± SD for cDNA samples independently isolated from 3 different experiments.

**Proximity Ligation Assays**

A Duolink proximity ligation assay (PLA) was used to quantify interactions between proteins less than ~40 nm apart, according to the manufacturer’s instructions. The method uses antibodies from two species (mouse and rabbit) to recognize two candidate proteins in situ. The antibodies are then recognized by secondary antibodies conjugated to complementary oligonucleotides, which are amplified to incorporate a fluorescent nucleotide (Texas Red) only if the pair of secondary antibodies are less than ~40 nm apart (Fredriksen et al., 2002; Koos et al., 2014) (Figure 5A).

Cells grown on fibronectin-coated 35-mm glass-bottom dishes were fixed at 20 °C (4% paraformaldehyde, 30 min), washed with PBS, permeabilized (0.25% Triton X-100, 5 min), and incubated with primary antibodies (16 hr, 4 °C). For ER-lysosome interactions, the primary antibodies were against VAP-A (ER) and either LAMP1 or Rab7 (both lysosomes), and for EGFP-IP3R1-lysosome interactions they were against GFP and either LAMP1 or Rab7. Incubations with Duolink PLA probe (anti-rabbit PLUS and anti-mouse MINUS), ligase and polymerase, and the washes between each step, were exactly as recommended by the manufacturer. Cells were then mounted in Duolink II mounting medium containing DAPI to label the nucleus. PLA products were visualized using an Olympus microscope with x60 or x100 objective, and spots were quantified using CellProfiler software. The specificity of the PLA reactions was confirmed by omission of either primary antibody (VAP-A or Rab7) and, for EGFP-IP3R1-lysosome measurements by using cells without EGFP-IP3R1 (Figure 5).

**Western Blots**

Cells isolated by centrifugation (600 xg, 2 min) were lysed in cold medium containing protease inhibitors (cOmplete, EDTA-free Protease Inhibitor Cocktail) and the supernatant (900 xg, 15 min) was used for western blotting. Proteins were separated (4%–8% RunBlue SDS gel, Expeadeon, San Diego, CA), transferred to a polyvinyl difluoride (PVDF) membrane using an iBLOT gel-transfer system (ThermoFisher), blocked in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.2% Tween-20 and 5% BSA for 1 hr, washed in the same medium, and incubated with primary antibody (16 hr, 4 °C) in the blocking buffer. After washing (3 x 5 min), the membrane was incubated with secondary antibody (1 hr, 20 °C), washed (3 x 5 min). Bands were visualized using ECL Prime western blotting detection reagent and a Syngene Pxi chemiluminescence detection system with GeneTools software.
QUANTIFICATION AND STATISTICAL ANALYSIS

We did not use power analyses to determine sample sizes. In all assays using multi-well plates, the positions of treatments were varied to avoid place-dependent systematic errors.

All statistical analyses used Prism, version 5. For analyses of concentration-effect relationships, non-linear curve-fitting to a Hill equation was used to provide values for pEC$_{50}$ (−log of the half-maximally effective concentration) and maximal response for each individual experiment. The individually determined pEC$_{50}$ values were then pooled for statistical analysis.

All results are presented as mean ± SD or SEM, as appropriate. Student’s t test (for 2 variables), and one-way or two-way ANOVA with Tukey’s multiple comparison test or Bonferroni post hoc test (more than 2 variables) were used for statistical analyses. The Kolmogorov-Smirnov normality test was used to determine whether frequency distributions deviated from normality (p < 0.05) (Figures 2F and 2G). Sample sizes (n) refer to independent experiments. p < 0.05 was considered significant. The tests used are reported in the figure legends.