INTRODUCTION

Injury to the plasma membrane poses a fatal threat to eukaryotic cells, owing to the immediate risk of osmotic lysis and subsequent leakage of cytosolic components to the extracellular environment. Thus, cells have developed efficient and rapid repair mechanisms to reseal a torn membrane and avoid cell death. Studies across a wide range of cell types spanning from Xenopus oocytes (1) to human breast cancer cells (2) show that wound healing takes place in the order of seconds to tens of seconds. This is comparable to the leakage rate of cytosolic fluid of a punctured cell, which, from a physical perspective, should be stopped within a similar time frame to prevent excessive loss of cell mass (3).

Accumulating evidence shows that plasma membrane repair mechanisms in eukaryotic cells rely on fundamental biological processes that are also used for other cellular functions, such as endocytosis (4), exocytosis (5), and membrane shedding (6, 7). The healing process also relies on various proteins including annexin family members. Upon injury and influx of Ca\(^{2+}\) into the cytoplasm, annexin proteins are activated and bind to anionic phospholipids in the plasma membrane in a Ca\(^{2+}\)-dependent manner (8). At the wound site, annexins facilitate repair by fusing wound edges (9), restricting wound expansion (10), and inducing curvature and constriction force (2), which help to pull the wound edges together for subsequent fusion. These initial repair processes usually occur within 10 to 45 s, depending on the extent of injury and cell type. However, once the cell has healed, it faces the issue of membrane restructuring to restore normal cell function (3). Here, restructuring defines the process by which the cell modifies the plasma membrane after initial resealing to restore its homeostasis.

Macrautophagy (hereafter autophagy) is a dynamic recycling system, which is activated as an adaptive response to a number of extracellular and intracellular stresses (11). It is implicated in a wide variety of physiological processes and serves as a major intracellular self-degradative process, where a portion of the cytoplasm is enclosed by a double membrane structure to form an autophagosome (12, 13). The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded. Some components of the canonical autophagy machinery are recruited to the phagosome during light chain 3 (LC3)–associated phagocytosis (LAP) (14). This process is triggered upon phagocytosis of pathogens or dead cells that engage cell-surface receptors, including Toll-like receptor (TLR) family members, resulting in the safe degradation of engulfed material upon fusion with a lysosome (14, 15). Unlike autophagy where the autophagosome is a double-membrane structure, the LC3-associated phagosome is composed of a single membrane, and its formation is characterized by being dependent on the class III phosphatidylinositol 3-kinase–associated protein Rubicon (Run domain Beclin-1 interacting and cysteine-rich containing) (14, 16, 17). Here, LC3 at the phagosome appears to enhance the fusion between phagosomes and lysosomes (18).

While the membrane wound healing response is gaining increasing focus, much less is known about how cells restructure their plasma membrane following the initial repair. In the absence of active restructuring, cells may accumulate dysfunctional areas on their membrane, which could affect the dynamics and general functionality of the cell. Here, we identify a previously unknown mechanism for membrane restructuring that consists of the induction of macroendocytic processes in association with LC3 and Rubicon around the repair site. As the accumulation of LC3 at the repair site is independent of unc-51 like kinase-1 (ULK1) but dependent on Rubicon, we propose that the observed process resembles LAP. However, it does not involve cargo engulfment. We therefore term the process LC3–associated macropinocytosis (LAM). LAM is triggered minutes...
after initial membrane resealing to internalize and degrade the damaged membrane and with that restore normal plasma membrane composition and function.

**RESULTS**

**LC3-positive vesicles accumulate at the repair site after plasma membrane injury**

To determine the kinetics of membrane wound healing, MCF7 breast carcinoma cells were exposed to injury by ablation laser and membrane integrity was measured using membrane-impermeable FM1-43 dye. In the presence of Ca$^{2+}$, cells were able to repair their plasma membrane within 20 to 30 s, as demonstrated by the decline of FM1-43 dye uptake seconds after injury (Fig. 1, A and B, left). On the other hand, cells injured in Ca$^{2+}$-deficient medium failed to repair and continued to take up the cell-impermeable dye (Fig. 1B, right). Thus, these cells possess a highly capable Ca$^{2+}$-dependent repair system that reseals holes within seconds in accordance with our earlier findings (2, 19).

Because autophagy is responsible for the degradation of cellular components and linked with multiple forms of cellular stress, we wondered whether an autophagy-associated response could be triggered after plasma membrane injury. To address this, we explored the behavior of one of the most used autophagy markers, microtubule-associated protein 1A/1B-light chain 3 (LC3B hereafter LC3) upon plasma membrane repair. MCF7 cells stably expressing the fusion protein enhanced green fluorescent protein (eGFP)–LC3 (MCF7 eGFP-LC3 cells) were used to investigate the LC3 response upon ablation laser–induced plasma membrane injury. Cells were also transfected with annexin A4 (ANXA4)–red fluorescent protein (RFP) to visualize effective wounding, because this protein is recruited to the site of injury within seconds in a Ca$^{2+}$-dependent manner (Fig. 1C) (2). LC3-positive puncta started to form at the repair site around 8 to 10 min after injury, in both MCF7 and HeLa cells (Fig. 1C, fig. S1A, and movie S1). This formation was spatially specific for the wounded area, because we did not observe a similar increase in LC3-positive vesicles in uninjured areas of the cell (Fig. 1D). Next, we investigated whether the formation of LC3-positive vesicles was dependent on the autophagy-related 7 protein (ATG7), which is an E1-like enzyme in the two ubiquitin–conjugation systems that are essential for the autophagosome biogenesis (20). Here, we used MCF7–ATG7 CRISPR knockout cells that showed a complete knockout of ATG7 and, furthermore, an inability to form the ATG5/12 complex that is required for the elongation and closure of the autophagosomal membrane (Fig. 1E) (autophagy deficient phenotype is characterized in fig. S2) (21). The MCF7–ATG7 CRISPR knockout cells were fully capable of repairing upon laser injury, as measured by membrane-impermeable FM4-64 dye uptake, which indicates that ATG7 is not required for the initial repair phase (Fig. 1F). However, cells deficient for ATG7 displayed a substantial reduced formation of LC3-positive vesicles around the repair site compared to control cells after damage (Fig. 1, G and H). Thus, injury-triggered formation of LC3-positive vesicles appears to be associated with components of the autophagy machinery, because ATG7 is an essential regulator of autophagosome assembly (20).

To further elucidate the origin of LC3-positive vesicles, MCF7 eGFP-LC3 cells were transfected with RFP-Rab5, a marker of early endocytosis (22) and monitored after different types of injury (by ablation laser or by squeezing cells from the top with a coverslip). We observed that most of the injured cells formed unexpectedly big Rab5-positive vesicles (1 to 3 μm in diameter) originating from the plasma membrane, starting around 2 to 5 min after injury (Fig. 2, A and B). Later, some Rab5-positive vesicles became completely LC3 positive (20 min) and appeared to colocalize with eGFP-LC3–positive vesicles (Fig. 2, A, B, and D, and movie S2). Expression of eGFP-LC3 together with early endosome antigen 1 (EEA1) (RFP-EEA1) revealed that large internalized vesicles eventually became RFP-EEA1 positive upon injury (Fig. 2C, 8 min), being readily detectable at the same time frame as the Rab5-labeled vesicles (at about 5 to 8 min after injury). Later, these RFP-EEA1–positive vesicles come in close proximity with eGFP-LC3 vesicles (Fig. 2C, 19 min), as previously observed with the Rab5-positive vesicles (Fig. 2A, 17 min). Moreover, some eGFP-LC3–positive vesicles and RFP-EEA1–positive vesicles colocalize and appeared to fuse (Fig 2C and movie S3).

In addition, we observed extensive injury-triggered membrane ruffling before vesicle formation. We have previously reported that depolymerization of the actin cytoskeleton at the injury site is followed by a rapid buildup of new actin structures (2, 19). To follow actin dynamics, MCF7 eGFP-LC3 cells were transfected with LifeAct-mCherry, a fluorescently tagged short peptide that binds to actin (23). As expected, we detected a rapid buildup of actin around the injury site (Fig. 2E), followed by the spatial and localized formation of actin-rich membrane protrusions (Fig. 2, F and G). Further analysis revealed that this cytoskeletal remodeling paved the way to the formation of LifeAct-positive vesicles starting around 2 to 5 min after injury (Fig. 2, H and I), following the same kinetics as the formation of Rab5- and EEA1-positive vesicles (Fig. 2, A and C). These actin-positive vesicles measure, on average, 0.5 μm in diameter (Fig. 2J). In contrast, the LC3-positive vesicles are smaller (with an average diameter of 1.8 μm, Fig. 2I) and form around 8 to 10 min after injury (Fig. 1C and movies S1 and S3).

To characterize the vesicles further, we coexpressed LifeAct-mCherry together with GFP-Rab35. The small Rab35 guanosine triphosphatase (GTpase) is involved in different endocytic processes, including endocytic recycling, and was reported to be required for remodeling of the actin cytoskeleton to form the phagocytic cup during phagocytosis (24). Internalized vesicles were enriched for both LifeAct-mCherry and GFP-Rab35 upon injury. GFP-Rab35 appeared enriched at the damaged membrane before accumulating at the limiting membrane of internalized vesicles (Fig. 2K and movie S4).

Similarly, Rab35– and LC3-positive vesicles were observed in HeLa cells after injury although less pronounced than in MCF7 cells (fig. S1, A and B).

In summary, these data show that two different pools of vesicles are formed after injury. Larger vesicles are formed earlier from the plasma membrane and are positive for Rab5, EEA1 (early endocytosis), actin, Rab35, and, occasionally, LC3. In contrast, later-forming vesicles are smaller and LC3 positive and eventually appear in close proximity to Rab5-positive vesicles.

**Internalized vesicles constrict and eventually shrink in the cytoplasm**

Next, we focused on the processing of the large internalized vesicles, which presumably are filled with extracellular fluid and appear extended in size (1 to 3 μm) as compared to other vesicular structures, which are processed in the cytoplasm. Following the fate of the formed vesicles in the cytoplasm using confocal time-lapse imaging.
revealed that most of the large vesicles positive for GFP–Rab35 disappeared from all focal planes within an hour (Fig. 3A and movie S5). In an attempt to model the behavior of these vesicles, we monitored vesicle fate by measuring their size with respect to time. Most vesicles appeared to shrink spontaneously after an initial period of nearly constant volume. The volume flux $J_V$ from the vesicles during the shrinking phase is defined as the change in volume per unit time, per unit surface area. For a spherical shell with radius $R(t)$, area $A(t) = 4\pi R^2$, and volume $V(t) = \frac{4}{3}\pi R^3$, one obtains

$$J_V = -\frac{1}{A} \frac{dV}{dt} = -\frac{dR}{dt} = -\dot{R}$$

\[1\]
Fig. 2. Macropinocytic processes are activated after plasma membrane repair. Representative sequential images of MCF7 eGFP-LC3 cells transfected with RFP-Rab5 that were subjected to either (A) laser injury (white arrow) (also see movie S2) or (B) coverslip squeezing (CS) and imaged for 20 min. Blue arrows point to vesicles, which are Rab5 positive during formation and become LC3 positive 13 to 20 min after injury. (C) MCF7 eGFP-LC3 cells transfected with RFP-EEA1 and exposed to laser injury. Blue and yellow arrows point to coaccumulating vesicles that appear to fuse. (D) Quantification of the number of RFP-Rab5, eGFP-LC3, and colocalized RFP-Rab5/eGFP-LC3-positive vesicles at the site of injury 15 min after laser injury. Mean ± SD from 15 cells obtained from three experiments. (E to J) Representative images and analysis of LifeAct-mCherry transfected MCF7 eGFP-LC3 cells after laser injury (white arrows). (E) Normalized fluorescence intensity of LifeAct-mCherry around the injury site, mean ± SD from five cells. (F) Representative images of actin-associated cell protrusions. White dashed area indicates the area used for quantification of actin-associated cell protrusions. (G) Quantification of the cell protrusion areas from six cells, 5 and 10 min after injury (mean ± SD). (H) Representative images demonstrating formation of LifeAct-positive vesicles (white dotted area indicates the area used for quantification of the number and diameter of LifeAct-positive vesicles). (I) Quantification of the number of LifeAct-positive vesicles per cell 0, 5, 10, 15, and 20 min after laser injury (mean ± SD), data from seven cells obtained from three experiments. (J) Quantifications of the diameter of LifeAct-positive vesicles 5, 10, and 15 min after laser injury (Mean ± SD from four individual cells). (K) Representative images of LifeAct-mCherry– and GFP-Rab35–transfected MCF7 cells after laser injury. Yellow arrows point to newly formed vesicles that are both LifeAct and Rab35 positive.
Under this approximation, the volume flux is simply minus the slope of the vesicle radius versus time. We measure an approximately linear decrease in vesicle radius versus time, corresponding to a nearly constant volume flux \( J_V \) with an average value of approximately 15 nm/s (Fig. 3, B to D).

The vesicle collapse likely arises from osmotic draining of solvent as described by Boroske et al. (25) and Freeman and Grinstein (26). However, to understand this process further, it is worthwhile to consider the details of the osmotic draining mechanism. In a classical description of osmotic shrinking of giant unilamellar vesicles...
as introduced by Boroske et al. (25), the outward volume flux is proportional to a difference Δc in osmotic concentration across the membrane

\[ J_V = -\dot{R} = PV_n\Delta c \quad (2) \]

where \( P \) is the permeability of the membrane to the solvent and \( V_n \) is the molar volume of the solvent, which, for water, is \( V_n = 18 \text{ ml/mol} \). The membrane permeability \( P \) was recently estimated for a 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol (90:10) bilayer to \( P = 15.7 \pm 5.5 \text{ mm/s} \) (27). In our analysis of shrinkage, we measure the mean volume flux to \( \langle \dot{V}_V \rangle = -dR/dt = 14.8 \pm 7.2 \text{ mm/s} \). Using the permeability estimate and Eq. 2, this leads to an osmotic concentration difference of \( \Delta c \approx 50 \text{ mM} \) and an osmotic pressure difference of \( \Pi = RT\Delta c \approx 1 \text{ bar} \) (\( R \) is the ideal gas constant \( R = 8.31 \text{ J/mol*K} \); \( T \approx 310 \text{ K} \) is the absolute temperature). Because vesicles cannot mechanically sustain an osmotic pressure difference of this magnitude, an osmotic concentration gradient is highly unlikely to have existed before the vesicles enter the shrinking phase.

The more likely mechanism of shrinking is induced by the gradual outward transport of osmolytes, followed by osmotically obliged water as reported by Freeman and Grinstein (26) (Fig. 3D). According to this model, the total number of osmolytes inside macropinosomes (mainly monovalent ions like Na\(^+\), K\(^+\), and Cl\(^-\)) is reduced because of channels and pump activity as exemplified by, e.g., members of the solute carrier (SLC) family and the mucolipin receptor (TRPML) family. The vesicular volume will concomitantly be reduced via osmotic transport of water out of the vesicles to balance the inside and outside osmotic concentrations. This model is supported by data where macropinosomes formed in physiological medium containing 150 mM NaCl shrink within minutes, whereas shrinkage is prevented when Na\(^+\) is replaced by a large and nontransportable cation (N-methylglucammonium\(^+\)) (26). Thus, we propose that osmotic draining of fluid from the vesicles drive the shrinkage process of large internalized vesicles after membrane damage and initial repair.

To investigate the fate of these large internalized vesicles after shrinkage, we tested whether vesicles eventually fuse with lysosomes. MCF7 eGFP-LC3 cells were injured by laser, and after 20 min or 6 hours of repair, the cells were fixed and stained for the lysosomal marker lysosome-associated membrane protein 2 (LAMP2). Large macropinosomes became positive for LC3, 20 min after injury, and a few subsequently colocalized with LAMP2 (Fig. 3E, top). Moreover, 6 hours after injury, both large and smaller eGFP-LC3–positive vesicles colocalized more prominent with LAMP2 (Fig. 3E, bottom). These data suggest that LC3–positive vesicles fuse with lysosomes and are processed further through the lysosomal degradation system.

**Macropinocytosis is triggered upon plasma membrane integrity**

Our findings support the phenomenon of injury-triggered macropinocytosis, which actin–driven extension of plasma membrane ruffles eventually form a cup-like structure that seals to form a large vesicle. Accordingly, we next asked whether macropinocytotic events are needed to restructure the plasma membrane after damage. Because Rab35 was reported to regulate actin-mediated pseudopod extension to form phagocytic cups (24), we first expressed a dominant negative mutant of GFP–Rab35 (GFP–Rab35S22N), locked in the guanosine diphosphate–bound, inactive form (24), and exposed cells to membrane injury by laser. While the wild-type GFP–Rab35 was significantly recruited to the limiting membrane of internalized vesicles 5 to 10 min after injury (Fig. 4A), GFP–Rab35S22N appeared in a more diffuse distribution around the damaged membrane, resulting in fewer internalized vesicles (Fig. 4, A and B). Moreover, MCF7 cells expressing the GFP–Rab35S22N mutant were more permeable to membrane-impermeable Hoechst dye upon laser injury, suggesting that Rab35 is important for macrointernalization and membrane integrity upon injury (fig. S4A). Second, we treated cells with dynasore—an inhibitor of the GTPase activity of dynamin, which should inhibit endocytosis in general by blocking vesicle constriction and fission (28). Dynasore treatment totally inhibited plasma membrane internalization resulting in lack of any visible LifeAct-mCherry–positive vesicles after injury (Fig. 4C). Likewise, formation of LC3-positive vesicles around the injury site was completely inhibited upon dynasore treatment (Fig. 4C and fig. S4B). In addition, dynasore-treated cells in the presence of membrane-impermeable Hoechst dye were more permeable after laser injury, incorporating more Hoechst dye intracellularly during the course of experiments (60 min) as compared to control cells (fig. S4, C and D). However, dynasore inhibits not only endocytosis through the inhibition of dynamin but may also have nonspecific effects by affecting lipid rafts, mammalian target of rapamycin complex 1 (mTORC1) activity, and F-actin (29, 30).

In search for a more specific inhibitor of macropinocytosis, we tested the selective Na\(^+\)/H\(^+\) exchanger blocker ethyl-isopropyl amiloride (EIPA), which has been extensively used as a canonical inhibitor of this process (31). Pretreatment of MCF7 cells with EIPA substantially inhibited macroendocytic events after laser damage, including the formation of vesicles positive for LifeAct-mCherry (Fig. 4, D and E), GFP–Rab35 (Fig. 4, F and G, and movies S6 and S7), and mCherry-Rab5 (Fig. 4, H and I). Similarly, expression of a dominant negative mutant of Rab5 (mCherry-Rab5DN) also inhibited internalization after damage and repair (Fig. 4, H and I). These findings point to macropinocytosis as the governing macroendocytic mechanism induced after plasma membrane damage and repair.

To address whether internalization of postinjured membrane via macropinocytosis is important for plasma membrane integrity, MCF7 cells were injured by laser, in the presence of EIPA and membrane-impermeable propidium iodide (PI) dye. Here, cells treated with EIPA were still able to repair after injury but were more permeable to PI dye (2 to 10 min) during the course of the experiment, suggesting that compromised macropinocytosis may affect membrane integrity after initial repair (Fig. 4J). Furthermore, we found that formation of new LC3-positive vesicles was drastically reduced after damage when macropinocytosis was inhibited by EIPA (Fig. 4, K and L), indicating that they are formed in response to macroendocytic events.

Cells became sensitized to cell death after mechanical injury (induced by glass beads and assayed after 3 hours) when macropinocytosis was inhibited by either EIPA (Fig. 4, M and N) or dynasore (Fig. 4O), indicating that macroendocytic processes are needed to cope with the aftermath of membrane damage to restore membrane integrity and ensure cell survival.

Because our laser injury approach generates damaged membranes, we next explored the possibility that the small LC3-positive vesicles could represent autophagosomes clearing damaged endosomes or lysosomes in proximity to the injury site. Endolysosomal membranes become galectin-positive upon damage because galectins
Fig. 4. Vesicle formation is compromised by inhibiting macropinocytosis and required for survival. (A) MCF7 cells expressing GFP-Rab35 (top) or GFP-Rab35S22N mutant (bottom) and exposed to laser injury (white arrow). Blue arrows, GFP-Rab35–positive vesicles. Also see movie S4. (B) Number of Rab35–positive vesicles per cell after laser injury in GFP-Rab35– or GFP-Rab35S22N–expressing cells. Mean ± SD. Ten cells per condition from three experiments. (C) Number of eGFP-LC3– and LifeAct-mCherry–positive vesicles per cell after laser injury (15 min) in cells treated with dynasore (80 µM) or DMSO (Ctrl). Mean ± SD, six cells from three experiments. See also fig. S4 (B to D). (D) Number of LifeAct-mCherry–positive vesicles per cell after laser injury in cells treated with EIPA (50 µM) or DMSO (Ctrl). Mean ± SD, nine cells per condition. (E) Images of MCF7 cells expressing LifeAct-mCherry and pretreated with either DMSO or EIPA before laser injury (white arrows). Blue arrows, LifeAct-mCherry–positive vesicles. (F) Number of Rab35–positive vesicles per cell after laser injury in GFP-Rab35–expressing cells treated with either EIPA (50 µM) or DMSO. Mean ± SD, 10 cells per condition, three experiments. (G) MCF7 cells expressing GFP-Rab35 and treated with EIPA before and after laser injury (white arrows). Also see movies S6 and S7. (H) Rab5–positive vesicles (blue arrows) upon laser injury in MCF7 cells expressing either mCherry-Rab5 (left) or mCherry-Rab5DN mutant (right) and treated with EIPA (50 µM) (middle). (I) Number of Rab5–positive vesicles per cell after laser injury in mCherry-Rab5– or mCherry-Rab5DN–expressing cells pretreated with either EIPA (50 µM) or DMSO (Ctrl). Mean ± SD, three experiments with a minimum of six cells per condition. (J) Membrane integrity upon laser injury (black arrow) in MCF7 cells pretreated with EIPA as measured by PI uptake. Mean ± SEM of normalized PI intensity, seven cells per condition from three experiments. (K) Same MCF7 eGFP-LC3 cells as in (H) (left, middle), demonstrating formation of eGFP-LC3–positive puncta after laser injury in Ctrl cells in contrast to EIPA-treated cells. (L) Quantification of newly formed eGFP-LC3–positive puncta per cell after laser injury in cells pretreated (30 min) with either DMSO (Ctrl) or EIPA (50 µM). Mean ± SD, three experiments with at least seven cells per condition. (M) MCF7 cells treated with either EIPA or DMSO and assayed for cell death (3 hours) after exposure to mechanical injury by vortexing cells in suspension with glass beads, followed by reseeding in the presence of EIPA or DMSO, and (N) corresponding images showing PI/Hoechst 33342–positive cells, after glass bead injury. Mean ± SD, of three independent experiments. (O) MCF7 cells pretreated (30 min) with dynasore (80 µM) and injured and assayed as in (M). Mean ± SD, of three independent experiments. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Two-way ANOVA with Bonferroni’s multiple comparisons test or Dunnett’s multiple comparisons.
recognize exposed intraluminal carbohydrate chains of endolysosomal glycoproteins. To address this, cells expressing mCherry–Galectin 3 (mCherry-Gal3) and eGFP-LC3 were damaged either by laser or as positive control by exposure to 1-leucyl-1-leucine methyl ester (LLOMe), which is converted into a membranolytic polymeric form in the endolysosome lumen by hydrolases (32, 33). While LLOMe, as expected, induced massive mCherry-Gal3– and eGFP-LC3–positive puncta that often colocalized (fig. S3A), we did not detect any major accumulation of mCherry-Gal3 puncta after laser injury (fig. S3B). Thus, injury-associated accumulation of eGFP-LC3 does not appear to be triggered in response to damaged endolysosomes.

Together, these experiments suggest that macroinocytosis is activated after injury and needed to restructure and, thus, maintain plasma membrane integrity. Moreover, LC3 vesicle formation is triggered in response to vesicle internalization.

**LC3 vesicle formation upon injury is independent of ULK1, ATG13, and WIPI2**

To gain insight into the nature of the LC3 vesicles formed as a consequence of plasma membrane internalization by macroinocytosis, MCF7 cells were depleted of the serine/threonine protein kinase ULK1 by small interfering RNA (siRNA) (Fig. 5B and fig. S5A). ULK1 protein kinase, in complex with autophagy-related 13 protein (ATG13) and FIP200, is a key initiator of canonical autophagy (34). As expected, control siRNA-transfected cells treated with the mTOR inhibitor, rapamycin, developed massive LC3 puncta, whereas ULK1-depleted cells failed to do so (Fig. 5A, left, and C). However, ULK1-depleted cells that were injured by laser were still able to form LC3 vesicles localized around the repair site and even to a larger extent than control siRNA-treated cells (Fig. 5A, right, and D). This difference could be driven by the increase in the pool of free LC3 when canonical autophagy is compromised, in combination with impaired autophagy turnover. Similarly, siRNA depletion of ATG13 (Fig. 5F and fig. S5B), which is indispensable for autophagy induction by the ULK1 complex, inhibited rapamycin-induced autophagy as visualized by a significant reduction in the formation of eGFP-LC3 puncta (Fig. 5, E and G). In contrast, accumulation of eGFP-LC3 after laser injury occurred independently of ATG13 and, as in ULK1-depleted cells, this accumulation exceeded that observed in control siRNA-treated cells (Fig. 5, H and I).

Next, we looked at the WD repeat domain phosphoinositide-interacting protein 2 (WIPI2), which plays a key role in canonical autophagy and functions downstream of ULK1. WIPI2 binds to the ATG12-ATG5-ATG16L1 complex that directly controls the elongation of the nascent autophagosomal membrane during starvation induced autophagy (35, 36). Coexpression of RFP-WIPI2 and eGFP-LC3 in MCF7 cells revealed no coaccumulation of RFP-WIPI2 and eGFP-LC3 puncta close to the damaged membrane. A few colocalization events were detected during the course of the experiments, but they did not appear around the repair site (Fig. 5J and fig. S6).

In contrast, MCF7 cells expressing p62/SQSTM1 (also known as sequestosome-1) fused to mCherry (mCherry-p62) and exposed to laser injury revealed a strong accumulation of mCherry-p62, which colocalized with eGFP-LC3 vesicles around the repair area (Fig. 5K and fig. S7). Because p62 is a cargo adaptor protein that localizes to sites of autophagosome formation, the results suggest that mCherry-p62 might interact with injury-associated substrates that are destined for degradation (37). Depletion of p62 by siRNA completely abolished local accumulation of eGFP-LC3 vesicles after injury, indicating that p62 is required for LC3 lipidation of vesicles derived from the damaged plasma membrane (Fig. 5, L to N). Next, we tested whether p62 siRNA knockdown compromises cell viability upon injury on the longer term, by damaging cells with glass beads and measuring cell death after 24 hours. Here, a small but statistically significant sensitization to cell death was detected in damaged cells depleted for p62 as compared to Ctrl (Fig. 5O).

Overall, these data suggest that plasma membrane injury triggers an ULK1–, ATG13–, and WIPI2-independent noncanonical autophagy process that requires p62 in response to induction of macroinocytosis.

**Rubicon localizes to the limiting membrane of macropinosomes and is required for LC3 accumulation**

Given the massive LC3 accumulation and induction of macroinocytosis upon plasma membrane injury, we hypothesized that this response could be related to LAP. This process is triggered by pathogens through TLR engagement (15). To test our hypothesis, MCF7 cells were siRNA-depleted for Rubicon—a protein that is required for LAP but not for canonical autophagy (Fig. 6B and fig. S5, A and C) (17). Rubicon-depleted cells were compromised in their ability to form LC3-positive vesicles as compared to control cells after injury (Fig. 6, A and C). However, Rubicon depletion did not affect the cells’ repair capacity (Fig. 6D) or their ability to internalize damaged membrane by macroinocytosis after injury (Fig. 6E). Moreover, as expected, the induction of canonical autophagy by the mTOR inhibitor rapamycin was not affected in Rubicon-depleted cells compared to control cells (Fig. 6, F and G).

Next, we sought to investigate whether Rubicon interacts with the two previously characterized pools of vesicles induced by plasma membrane damage. First, we focused on the Rab5- and Rab35-positive larger vesicles. MCF7 cells expressing GFP-Rubicon together with RFP-Rab35 showed a significant recruitment of GFP-Rubicon to the limiting membrane of internalized Rab35–RFP–positive macropinosomes upon injury induced by cell squeezing with glass coverslip (Fig. 6H). Likewise, internalized Rab5–positive macropinosomes, as well as smaller Rab5–vesicles, as a result of laser injury became positive for GFP-Rubicon. Here, smaller RFP-Rab5/GFP-Rubicon–positive vesicles resembled small macropinosomes/endoosomes that accumulated in proximity to larger macropinosomes in a similar pattern as observed for large LC3-positive vesicles (Fig. 6, I and J, and movie S8).

Together, our results suggest that MCF7 cells upon plasma membrane injury use macroinocytosis to internalize and restructure the damaged membrane, which is associated with a noncanonical autophagy process similar to LAP. We term this process LAM.

**DISCUSSION**

Only limited knowledge exists about how cells cope with plasma membrane disruptions after initial repair. Here, we propose that the healing response requires an immediate repair response phase activated within seconds and a subsequent restructuring phase, which involves removal of damaged membrane around the injury site by macroinocytosis. Our results show that the restructuring phase is important for cell survival upon injury, because blocking macroinocytosis by inhibitors such as EIPA and dynasore sensitizes cells to death induced by membrane damage. We find that in response to
Fig. 5. Activation of LC3 response upon injury is independent of ULK1 and ATG13 but requires p62. Analysis of LC3 response in MCF7 eGFP-LC3 cells. (A) Representative images of cells transfected with Ctrl or ULK1 siRNA (72 hours) and exposed to rapamycin treatment (200 nM) or laser injury (white arrows). (B) Corresponding immunoblot of lysates after siRNA transfection (72 hours) (Hsp90, loading control). (C) Number of LC3 puncta per cell after treatment with rapamycin (200 nM) in cells transfected with Ctrl or ULK1 siRNA (72 hours). At least eight cells per experiment were analyzed from three experiments. Mean ± SD. (D) Quantification of eGFP-LC3 fluorescence around the repair site following laser injury (black arrow) in cells transfected with ULK1 or Ctrl siRNA (72 hours). Mean ± SEM of normalized eGFP-LC3 intensity from eight cells per condition from three experiments. (E) Images of LC3 puncta formation after treatment with rapamycin (30 min) in cells transfected with Ctrl or ATG13 siRNA (72 hours) and (F) corresponding immunoblot after siRNA transfection (72 hours) (GAPDH, loading control). Extended blot is shown in fig. S5C. (G) Number of LC3 puncta per cell after rapamycin (30 min) in cells transfected with Ctrl or ATG13 siRNA (72 hours). Mean ± SD, 12 cells per condition from three experiments. (H) LC3 puncta formation after laser injury (white arrows) in cells transfected with Ctrl or ATG13 siRNA (72 hours). (I) Quantification of eGFP-LC3 fluorescence around the repair site following laser injury (black arrow). Mean ± SEM of normalized intensity from nine cells per condition from three experiments. (J) Cells expressing RFP-WIPI2 before and after laser injury (white arrow). Blue arrows, eGFP-LC3–positive puncta. Yellow arrows, puncta positive for RFP-WIPI2 and eGFP-LC3. Individual channels are shown in fig. S6. (K) Sequel images of MCF7 eGFP-LC3 cells expressing mCherry-p62 and exposed to laser injury (white arrow). Yellow arrows, puncta positive for both mCherry-p62 and eGFP-LC3. Individual channels are shown in fig. S7. (L) Quantification of eGFP-LC3 puncta around the injury site (black arrow) in cells depleted for p62 by siRNA (72 hours). Mean ± SEM, from 14 cells per condition from three experiments. (M) Corresponding immunoblot from p62 siRNA transfection (72 hours) (Hsp90: loading control). Extended blot is shown in fig. S5B. (N) eGFP-LC3 puncta formation after laser injury (white arrow) in cells depleted for p62 as compared to Ctrl siRNA. Note that p62 depletion induces some nonspecific accumulation of eGFP-LC3 without injury. (O) MCF7 cells depleted for p62 by siRNA (72 hours) and exposed to glass bead injury (by vortex) and then reseeded and assayed for cell death (24 hours) by PI/Hoechst 33342 exclusion. Mean ± SD, of five independent experiments. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Two-way ANOVA with Bonferroni’s multiple comparisons test or unpaired t test of AUC values with Welch’s correction.
Fig. 6. Activation of LC3 response after injury requires Rubicon. (A) Representative sequential images of LC3 vesicle formation after laser injury (white arrows) in MCF7 eGFP-LC3 cells transfected with either Ctrl (top) or Rubicon siRNA (bottom) (72 hours) and (B) corresponding immunoblot of lysates after transfection (72 hours). Extended blot is presented in fig. S5C. (C) Quantification of eGFP-LC3 fluorescence around the repair site following laser injury (black arrow) in cells transfected with either Ctrl or Rubicon siRNA (72 hours). A total of 24 cells were analyzed per condition from three experiments. (D) Plasma membrane repair kinetic upon laser injury in MCF7 cells transfected with Ctrl or Rubicon siRNA (72 hours) as measured by normalized FM1-43 cytoplasmic dye levels from 14 cells per condition obtained from three experiments. Mean ± SEM. (E) Number of Rab35-positive vesicles per cell at indicated time points after laser injury in RFP-Rab35-expressing cells transfected with Ctrl or Rubicon siRNA (72 hours). Mean + SD, five cells per condition from three experiments. (F) Number of LC3 puncta per cell after rapamycin treatment (200 nM, 30 min) in cells transfected with Ctrl or Rubicon siRNA (72 hours). Three experiments with eight cells per condition were analyzed, mean + SD. (G) Representative images of eGFP-LC3 puncta formation after treatment with rapamycin (200 nM, 30 min) in cells transfected with Ctrl or Rubicon siRNA (72 hours). (H) Images of MCF7 cells transfected with GFP-Rubicon and RFP-Rab35 uninjured and after coverslip squeezing (CS). Blue arrows, Rab35-positive vesicles that become Rubicon positive 15 min after injury. (I) Images of MCF7 cells expressing GFP-LC3 and GFP-Rubicon upon laser injury. Blue arrows point to vesicle colocalization. (J) Representative sequential images of MCF7 cells expressing GFP-Rubicon and RFP-Rab5 before and after laser injury (white arrow). Blue arrows point to both small and large vesicles, which are Rab5 positive during formation and become Rubicon positive 5 to 8 min after injury. **P ≤ 0.01. Unpaired t test of AUC values with Welsh’s correction.
localized membrane damage, macropinocytosis has spatiotemporal specificity and that this process is mainly characterized by the accumulation of LC3 around the injury site. This process is dependent on Rubicon and p62 and shares similarities with LAP (14, 15).

Our data show that plasma membrane injury triggers extensive buildup of actin around the injury site, followed by the spatial and localized formation of actin-rich membrane protrusions and membrane ruffling before vesicle formation. Large-forming vesicles then originate from the wounded plasma membrane. They are first positive for Rab35 and actin at the membrane and then become positive for the endocytosis markers Rab5 and EEA1 (Fig. 7). This process can be inhibited by expressing dominant-negative mutants of Rab5 and Rab35 and by treating cells with the macroendocytosis inhibitor EIPA. Internalized vesicles eventually become positive for Rubicon but are not dependent on Rubicon for their formation. We noticed that the mode of injury affects the size of internalized macropinosomes. While laser injury creates internalized macropinosomes of varying sizes (0.5 to 5 μm), mechanical injury (inflicted by squeezing with glass coverslips on the top of cells) induces mainly large uniform macropinosomes (>3 μm). The difference may reflect that coverslip squeezing induces a broader and more uniform pressure and injury to the cells, whereas the ablation laser creates membrane holes with more fragmented wound edges.

Once internalized, most large macropinosomes undergo shrinkage in the cytoplasm, which could be driven by osmotic draining induced by the outward transport of osmolytes that is followed by osmotically obliged water, as previously suggested (26). The shrinkage process from micrometer- to nanometer-size vesicles supposedly enables further processing of macropinosomes from the injured membrane through lysosomal degradation and recycling (Fig. 7).

There seems to be two events occurring after injury that both involve noncanonical autophagy and are influenced by the size of internalized vesicles. On the one hand, small LC3-positive vesicles are generated more frequently and are positive for p62 and dependent on Rubicon and p62 for their formation. These vesicles are made independently of ULK1, ATG13, and WIPI2 and are in close proximity to both small and large macropinosomes. Furthermore, these smaller vesicles are positive for endosomal markers RFP-Rab5 and GFP-Rubicon. We propose that small macropinosomes/endosomes formed in response to membrane injury are targeted for lysosomal degradation via noncanonical autophagy after membrane injury (38). These small LC3 vesicles do not seem to be formed as a response to damaged endolysosomes, because we did not detect any major accumulation of mCherry-Gal3 puncta or colocalization of mCherry-Gal3 puncta and eGFP-LC3 puncta upon damage.

On the other hand, LC3 and Rubicon appear to be directly associated to large macropinosomes (>2 μm; positive for Rab5, Rab35, and actin) after damage, through the noncanonical pathway resembling LAP (17). It has been shown that macropinosomes can be targeted for LC3 lipidation independent of vesicle formation, which

![Fig. 7. Proposed model for membrane restructuring by LAM after initial plasma membrane repair.](image-url)
our data support (38, 39). However, macropinocytosis does not involve cargo engulfment, as is known from LAP where phagocytosis of pathogens or dead cells engage cell-surface receptors and are eaten.

LAM seems to share some of its machinery with canonical autophagy but is functionally distinct and is not affected by mTOR modulation (15, 40). Moreover, macropinosomes are composed of a single membrane in contrast to classical autophagy where the autophagosome is surrounded by a double membrane structure (41). In line with reports regarding LAP, our results show that LC3 recruitment requires some components of the classical autophagy machinery including ubiquitin-like modifier-activating enzyme ATG7 (14, 15). While LAP was discovered in macrophages exposed to particles that engage TLRs (15), we find that a related process can be triggered by plasma membrane injury. We observe LC3 being conjugated to vesicles at the repair site around 8 to 10 min after injury; this is similar to the timing described for LC3 recruitment during LAP (<15 min after internalization) (15). Because lipidated LC3 proteins are known to promote hemifusion between membranes in vitro (42), it may enhance fusion of phagosomes/macropinosomes with lysosomes during LAP/LAM. This is further supported by data showing that when LC3-II is cleaved from membranes by expressing the Legionella effector protein RavZ, the fusion with lysosomes during LAP is compromised (17, 43), pointing to the role of lipidated LC3 in promoting phagosome-lysosome fusion.

Earlier studies showed that endocytosis is required for membrane rescaling after permeabilization with bacterial pore-forming toxins, e.g., streptolysin O (SLO), by directly removing SLO-containing pores from the plasma membrane (4). However, a recent study using a specific real-time reporter system suggests that endocytosis is not directly involved in pore removal from the plasma membrane but more likely is involved in restructuring the membrane in response to damage (44). Moreover, because endocytosis triggered by pore-forming toxins occurs faster than LAP, it remains to be examined whether this process eventually involves LC3-II-associated degradation.

Together, our results suggest that cells actively replace parts of their plasma membrane upon injury and repair through macropinocytosis. The initial step to internalize damaged membrane by macropinocytosis appears to be the critical process for cell survival upon injury during restructuring. We propose that macropinocytosis enables cells to better cope with plasma membrane disruptions by removing wound areas containing both damaged membrane and proteins after initial repair. Thus, LAM supports the restructuring phase of the injury response, to fully reestablish plasma membrane integrity and homeostasis upon damage.

**METHODS**

**Cell culture and treatments**

Human breast carcinoma cell line MCF7 and HeLa human cervix carcinoma cells were cultured in RPMI 1640 supplemented with 6% heat-inactivated fetal bovine serum (FBS) and 0.25% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were regularly tested and found negative for mycoplasma as analyzed by polymerase chain reaction and Hoechst staining. The MCF7 S1 clone and its derivative expressing eGFP-LC3B have been described previously (45, 46). MCF7 eGFP-LC3B ATG5 as well as ATG7 CRISPR KO and the corresponding nontargeted control cells were generated by lentiviral transduction, followed by single-cell clone selection (47) using previously described CRISPR constructs provided by K. Ryan (48). Live-cell imaging was performed in cell-imaging media: colorless RPMI 1640 supplemented with 2 mM GlutaMax, 6% FBS, 0.25% penicillin-streptomycin, and 25 mM Heps (Gibco, Sigma-Aldrich).

Autophagy was induced using either 200 nM rapamycin (Sigma-Aldrich) for 30 min or 100 nM for 4 hours or induced by amino acid starvation by incubation of cells in Hanks’ balanced salt solution (Gibco, 14025092) for 4 hours. Autophagy was blocked using 4 nM concanamycin A for 4 hours (Santa Cruz Biotechnology). Lysosomal damage was induced using 2 mM LLOMe (Santa Cruz Biotechnology). Endocytosis inhibition was performed by preincubation with 80 μM dynasore (Sigma-Aldrich) for 30 min before imaging. Macropinocytosis inhibition was performed by preincubation with 50 μM EIPA (Sigma-Aldrich) for 30 min before cells were subjected to wounding and membrane integrity experiments. Control samples were treated with dimethyl sulfoxide (DMSO).

**Plasma membrane wounding experiments and membrane integrity assays**

**Laser injury**

Cells grown in MatTek imaging–culture dish were kept at 37°C in cell imaging media, and a 2-μm circular region was irradiated using a 355-nm ultraviolet ablation laser (Rapp OptoElectronic) with the following settings: 2.6% power, 200-Hz repetition rate, pulse energy > 60 μJ, and pulse length < 4 ns. Images were acquired with the inverted microscope Eclipse Ti-E (Nikon) paired with the UltraVIEW VoX Spinning Disk (PerkinElmer) using the 63× objective. Cells were imaged 4 to 30 s starting before injury and continuing for 5 to 60 min following injury. Control of hardware and intensity measurements were performed with PerkinElmer’s Volocity software.

**Glass coverslip injury**

Cells were injured by placing a coverslip on top of cells seeded in a MatTek imaging–culture dish. Then, cells were injured by gently squeezing the coverslip down on the cells with a pipette tip and imaged. Vesicles and eGFP-LC3 puncta were counted manually. Plasma membrane integrity was measured by monitoring uptake of dye as a change in fluorescence during the course of imaging for the dyes: FM1–43 (Invitrogen; 1.6 μM), FM4–64 (Invitrogen; 2.5 μM), PI (Sigma Aldrich; 0.5 μg/ml), or Hoechst 33258 (Sigma Aldrich; 2.5 μg/ml).

**Glass bead injury**

Cells were transfected with 25 nM siRNA for 72 hours before glass bead injury or pretreated for 30 min with 20 or 30 μM EIPA or 80 μM dynasore (Sigma-Aldrich). Control cells were treated with DMSO. A total of 7 × 10⁴ cells were then mixed with 250 mg of 425- to 600-μm glass beads (Sigma-Aldrich, G8772) in Eppendorf tubes, followed by disruption of cells by vortex for 0, 30, or 60 s. After injury, 5000 or 10,000 cells were plated per well in 96-well plates and incubated at 37°C for 24 hours for cells transfected with siRNA or for 3 hours for cells treated with EIPA or dynasore to measure long-term effects on cell viability after injury. Membrane integrity was measured with PI (0.2 μg/ml) and Hoechst 33342 (Sigma Aldrich; 5.7 μg/ml) using a Celigo cytometer (Brooks Life Science Systems) according to the manual and analyzed for total and dead cells using Celigo software version 2.1.

**Plasmids and siRNA transfections**

ANXA4-tRFP had been generated by cloning ANXA4 (Origene, #RG203229) into the pCMV6-AC-tRFP vector (Origene, #PS100034) (2).
The eGFP-LC3 was a gift from T. Yoshimori (49). The mRFP-Rab5 plasmid was a gift from A. Helenius (Addgene, plasmid #14437) (50). The mCherry-Rab5 plasmid was a gift from G. Voeltz (Addgene, plasmid #49201) (51). TagRFP-T-EEA1 plasmid was a gift from S. Corvera (Addgene, plasmid #42635) (52). The mCherry-Rab5DN(S34N) plasmid was a gift from S. Grinstein (Addgene, plasmid #35139) (53). The GFP-Rab35WT and GFP-Rab35S22N inactive plasmids were a gift from Peter McPherson (Addgene, plasmid #47424 and #47426) (54). The RFP-Rab35 plasmid was a gift from J. Presley (55). LifeAct-mCherry plasmid was a gift from H. Meyer (Addgene, plasmid #47426) (55). The mCherry-Rab5DN(S34N) (Addgene, plasmid #42635) (56). Expression plasmid with OFPspark/RFP N-terminal tag containing human WIP12 cDNA was purchased from Sino Biological Inc. (HG14898-ANR). The mCherry-p62 plasmid was a gift from T. Johansen (57). The eGFP-Rubicon and mRFP-LC3 plasmids were a gift from T. Yoshimori (Addgene, plasmid #21636 and #21075) (58). Cells were transiently transfected with the indicated plasmids using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's instructions, omitting the Plus Reagent, and live-cell imaging experiments were performed the day after transfection.

Reverse siRNA transfection was performed using Oligofectamine Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. The siRNA-containing medium was replaced after 24 hours, and the analysis was performed after 72 hours. SiRNAs were purchased from Sigma-Alrich unless otherwise stated. ULK1 siRNA (5'-CCCUUUGCCUUAAUUGA-3') was used at 25 nM concentration, Rubicon siRNA (5'-GUCAGAUGAGGAUGACAU-3') was used at 50 nM, ATG13 siRNA (5'-GUCUGUGAAGACUCUCAGA-3') was used at 25 nM, p62 siRNA (5'-GGCUGAAAGGAAGCUCCUU-3') was used at 25 nM, and AllStar Negative Ctrl siRNA (1027281, Qiagen) was used accordingly.

Immunoblotting
Cells were lysed in Laemmli sample buffer [125 mM tris (pH 6.7), 20% glycerol, 140 mM SDS, and 0.3 μm bromophenol blue] supplemented with 0.1 M dithiothreitol, protease inhibitor cocktail (Roche, 4693124001), and phosphatase inhibitor (Roche, 4906837001). Cell lysates were boiled for 5 min and separated by SDS-polyacrylamide gel electrophoresis using precast 4 to 15% TGX Gel (Bio-Rad), followed by transfer to nitrocellulose membranes (Bio-Rad) using Trans-Blot Turbo transfer system. Novex Sharp Protein Standard (Invitrogen LC5800) was used to evaluate the molecular weights of proteins from the gels. After blocking in PBS containing 0.1% Tween-20 (PBST) and 5% milk, membranes were incubated with primary antibodies in PBST/5% BSA/0.1% NaN3 at 4°C overnight. Primary antibodies raised against human ATG5 (Cell Signaling Technology, 12994; 1:1000), ATG7 (Cell Signaling Technology, 8558S, 1:1000), Hsp90 (BD Biosciences, 610418; 1:4000), Rubicon (Cell Signaling Technology, 7151; 1:1000), ATG13 (Cell Signaling Technology, 13273; 1:1000), ULK1 (Cell Signaling Technology, 8054; 1:2000), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, ab189095; 1:7500), p62 (Progen Biotech, GP62-C; 1:1000), and LC3B (Cell Signaling Technology, 3868 (D11); 1:1000) were used. This was followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibodies [anti-rabbit immunoglobulin G (IgG), Vector Laboratories, PI-1000; anti-mouse IgG, Dako, P0260; both 1:5000] for 1 hour at room temperature. Chemiluminescent signals (Clarity Western ECL Substrate, Bio-Rad) were detected with Luminescent Image Analyzer (LAS-1000Plus, Fujifilm, Tokyo, Japan).

Immunocytochemistry
MC17 eGFP-LC3 cells grown in MatTek imaging-culture dishes were subjected to laser injury as described previously. Twenty minutes or 6 hours after injury, cells were fixed with 4% paraformaldehyde and permeabilized with ice-cold methanol and blocked in Dulbecco’s PBS/5% goat serum (Dako, X0907)/1% BSA/0.3% Triton X-100 and stained with primary antibody against human LAMP2 (DSHB H4B4; 0.8 μg/ml). Samples were incubated with appropriate Alexa Fluor 568–conjugated antibody (Invitrogen, A10037; 1:1000), and images were taken with the inverted microscope Eclipse Ti-E (Nikon) paired with the UltraVIEW VoX Spinning Disk (PerkinElmer) using the 63× objective.

Quantification of vesicle size
Quantification of vesicle size was done using image processing in MATLAB (MathWorks). First, the image sequence from the confocal microscope was cropped around the region of a single vesicle and smoothed using adaptive noise-removal filtering (Wiener2). The smoothing box was well below the image resolution. The image stack was then binarized to black/white using a constant threshold, and the boundary and position of the vesicle were tracked with respect to time until disappearance of the vesicle. The vesicle radius was last determined by equating the measured vesicle area to the area of a circle.

Statistical analysis
The statistical significance of results was analyzed using GraphPad Prism version 8.1.1 (GraphPad Software Inc.). The datasets from vesicle counts and glass bead injury were analyzed by one-way or two-way analysis of variance (ANOVA) test, followed by Dunnett’s or Bonferroni’s multiple comparisons tests (α = 0.05). The fluorescence intensity changes over time were analyzed by first calculating the mean of the normalized intensity values as a function of time. Then, the null hypothesis that changes in fluorescence intensities over time are the same for different experimental groups was performed by first quantifying the area under the curve (AUC) for each cell and then applying a two-tailed t test on the AUC values from the two experimental groups. A paired t test was performed when the measurement came from the same cell. In case of unpaired t test, Welch’s correction was last determined when the variances between the experimental groups were significantly different as determined by the F test.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/27/eabg1969/DC1

View/request a protocol for this paper from Bio-protocol.

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