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Impact of the endosomal escape activity of cell-penetrating peptides on the endocytic pathway

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Abstract

Cell-penetrating peptides (CPPs) are routinely used for the delivery of macromolecules into live human cells. To enter the cytosolic space of cells, CPPs typically permeabilize the membrane of endosomes. In turn, several approaches have been developed to increase the endosomal membrane permeation activity of CPPs so as to improve delivery efficiencies. The endocytic pathway is however important in maintaining cellular homeostasis and understanding how endosomal permeation impacts cells is now critical to define the general utility of CPPs. Herein, we investigate how CPP-based delivery protocols affect the endocytic network. We detect that in some cases, cell penetration induces the activation of Chmp1b, Galectin-3, and TFEB, components of endosomal repair, organelle clearance, and biogenesis pathways, respectively. We also detect that cellular delivery modulates endocytosis and endocytic proteolysis. Remarkably, a multimeric analog of the prototypical CPP TAT permeabilizes endosomes efficiently without inducing membrane damage responses. These results challenge the notion that reagents that make endosomes leaky are generally toxic. Instead, our data indicates that it is possible to enter cells with minimal deleterious effects.

Keywords

membrane repair; endosomal escape; delivery agents; autophagy; biogenesis; cell recovery; TAT; cell-penetrating peptides

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Supporting Information Available:

This material includes the method section and supplementary figures and is available free of charge *via* the Internet.

Introduction

Delivery vectors that transport macromolecules into live human cells are required for numerous applications in cell biology or biotechnologies. These reagents also have therapeutic potential as they may enable the development of biologics that modulate intracellular targets.¹ Examples include cationic lipids or engineered viruses, both of which are optimized for the delivery of recombinant DNA.^{2, 3} The prospect of delivering a wider range of biomolecules with greater efficiencies or control has led to the development of new reagents, like cell-penetrating peptides (CPPs). CPPs can deliver DNA as well as peptides, proteins, and RNAs.^{4, 5} To enter cells, CPPs often accumulate inside endosomes after endocytic uptake and then escape from endosomes via processes that often remain unclear.⁶ Endosomal escape requires the disruption of endosomal membranes so as to allow the egress of delivered cargos into the cytosolic space of cells. In principle, the number of macromolecules that a delivery reagent can transport into the cytosol of a cell is proportional to the number of endosomal organelles permeabilized, and to the extent by which the membrane of these organelles is destabilized. Given that endocytic organelles are important to a variety of cellular processes, the disruption of the integrity of their membranes by delivery agents is expected to have various deleterious effects.⁷ This raises questions of whether efficient delivery reagents are necessarily harmful or toxic, and consequently, of whether there is a limit to how much material can be delivered into cells. Recently, several reports have highlighted how lysosomotropic agents, transfection reagents, particulate debris and pathogens induce membrane repair pathways upon disruption of endosomal membranes.^{8–13} These pathways can protect cells from the harmful consequences of membrane damage and help them reestablish homeostasis.¹⁴ Interestingly, by continually counteracting the damage performed by membrane-disrupting species, these responses may also inhibit the translocation of endocytosed toxins or pathogens.¹⁵

Herein, we investigate CPP-based delivery agents and establish their impact on the endocytic pathway. Our motivation for this study was three-fold. First, we aimed to examine whether membrane damage responses inhibit the cytosolic translocation of CPPs or mask some of their potentially deleterious effects. Second, the triggering of endosomal membrane damage responses is used as an assay to screen and identify endosomolytic delivery agents from chemical libraries.¹⁶ This type of proxy assay could in principle facilitate the development of improved CPPs. However, the correlation between membrane damage and cytosolic egress of cargos has not been clearly established, potentially leading to misleading results. Finally, understanding the impact that cell penetration has on cells is critically important to define the applications that are adequate when using delivery approaches. For example, the delivery of transcription factors for the reprogramming of stem cells, as well as the delivery of gene-editing complexes, is useful and controllable as long as the delivery process itself does not cause major transcriptional responses in manipulated cells.^{17–19} Similarly, using delivered probes to monitor cellular processes is only meaningful if cellular homeostasis is not disrupted by the delivery itself. In this context, our goal was to determine how cells respond to the disruption of endosomal membranes during CPP-based delivery protocols and establish how cells recover from this process. We exploit the fact that several CPP-based

reagents have recently shown a broad range of endosomal escape activities, thereby giving for the first time the opportunity to establish a spectrum of possible responses.^{20–22}

Results

Reagents used and experimental design.

To establish the interplay between delivery efficiency, endosomal escape, and cellular responses to membrane damage, we chose a subset of delivery agents related to the prototypical CPP HIV TAT peptide (Figures 1 and S1). TAT is a commonly used CPP endocytosed by cells at low micromolar incubation concentrations.²³ Due to its relatively poor endosomal escape activity, TAT remains mostly trapped inside endosomes.²⁴ We therefore expect that this peptide would represent a CPP with low membrane damage activity. Labeling of TAT with the fluorophore tetramethylrhodamine permits microscopy visualization of the peptide but also confers the TMR-TAT conjugate with a photolytic activity.²⁰ In particular, upon irradiation with high intensity visible light, the fluorophore moiety produces reactive oxygen species (ROS). In turn, the TAT moiety targets the TMR and the generated ROS to endosomal membranes. Lipids in endosomal membranes are oxidized and endosomes subsequently rupture, thereby releasing their content. This light-induced activity can be exploited to improve cellular delivery efficiencies, as described in the strategy known as Photo Chemical Internalization (PCI).²⁵ In this study, we envisioned that light would provide a useful trigger to induce endosomal membrane damage in a time-controlled manner.

An alternative way to enhance the endosomal escape of TAT consists of conjugating multiple copies of this peptide to create constructs presenting a high local concentration of arginine residues. A reagent that follows this strategy is the disulfide bonded dimer dFTAT.²¹ dFTAT escapes from endosomes efficiently and localizes in the cytosol and nucleus of cells.²⁶ dFTAT promotes the cytosolic delivery of a wide variety of molecules, from small organic molecules and peptides to large proteins (25–150 kDa) and nanoparticles (100 nm in diameter).^{21, 27} The successful cytosolic delivery of large cargos implies that substantial membrane disruptions occur. In this study, we therefore expect dFTAT to serve as a model for CPPs that disrupt and modify endosomal membranes extensively.

UNC7938 is a small molecule that can deliver nucleic acid payloads into cells *in vitro* and *in vivo*.²⁸ UNC7938 also enhances the endosomal escape and cytosolic delivery of TAT-like CPPs. For instance, while the effect of UNC7938 is not readily noticeable when dFTAT enters cells effectively on its own (e.g. at 5 μ M incubation), the small molecule greatly improves the cell penetration of the peptide under conditions where the peptide alone would otherwise remain trapped inside endosomes (e.g. at 1 μ M incubation). Overall, UNC7938 primes the endosomal membrane for CPP-mediated leakage and this can be exploited to achieve delivery of nucleic acid and protein cargos under conditions where CPPs alone fail.²² Given the boost in endosomal escape activity provided by UNC7938, we envision that this molecule may increase the damage of endosomal membranes and potentially trigger cellular responses that CPPs alone may not elicit. Finally, Lipofectamine and L-leucyl-L-leucine methyl ester (LLOME) were chosen as controls. Lipofectamine is a broadly used transfection agent that differs from CPPs in structure, charge, and type of cargo delivered.

LLOME is endocytosed by cells and converted into a membranolytic polymer by hydrolases present in the endocytic pathway.²⁹ LLOME is not a delivery agent but this molecule is generally used as a model for the study of endosomal membrane repair, and will be used herein as a positive control.^{8, 9} Cell incubation times and protocols, which vary based on the experimental design, are summarized for all assays in supplemental information (Figure S2).

CPP-induced endosomal membrane leakage

Based on the literature, the delivery reagents chosen for this study are expected to induce endosomal membrane leakage with various efficiencies. However, the quantification of delivery efficiencies often uses a wide variety of cargos and indirect assays, making side-by-side comparisons difficult. In order to directly establish the endosomal membrane permeabilization activities of the reagents used herein, we chose several assays that measure the escape of molecules from the lumen of endosomes. We first focused on detecting the endosomal escape of H^+ and Ca^{2+} , as these ions are more concentrated inside endosomes than in the cytosol of cells. We found that dTAT and dTAT/UNC7938 may partially release both ions (Figure S3). However, slow release kinetics and transport mechanisms that reestablish homeostasis may mask some of these effects. To circumvent these issues, SNAP-S488, Fl-k5, and AF488-H1 were used as reporters of endosomal release. SNAP-S488 (842 Da) is a membrane impermeable small fluorophore, Fl-k5 (1016 Da) is a penta-lysine peptide that is protease-resistant, and AF488-H1 (21 kDa) is the labeled Histone 1 protein. These probes accumulate inside endosomes along with delivery agents, escape endosomes upon sufficient leakage, and accumulate within the nuclei of cells (Figure 2).²¹ Probes were co-incubated with delivery agents and the intracellular distribution of the probes was quantified based on the number of cells displaying nuclear fluorescence. In the case of LLOME, Lipofectamine, TMR-TAT, and UNC7938, fluorescence microscopy showed endosomal entrapment of probes, suggesting that, if endosomal leakage happens, it is below the detection limit of our assays. The photolytic activity of TMR-TAT in the presence of lights shows a redistribution of Fl-k5. The efficiency of this process is low because the power of the light source was limited to avoid cytotoxic effects. In contrast, dTAT or the dTAT/UNC7938 cocktail redistributes the three probes to the cytosol and nucleus in a majority of cells in a culture. This is also observed when endosomes are preloaded with probes, confirming that endosomal entrapment precedes endosomal escape (Figure S4). Notably, cell penetration of dTAT or dTAT/UNC7938 was non-toxic to cells (unlike LLOME which leads to a delayed loss of viability, Figure S5)

Triggering of endosomal membrane damage and repair responses

We next evaluated whether cells engage in endosomal membrane damage responses. These responses include repair of membrane defects, clearance of damaged organelles by autophagy, and replacement of organelles by de novo biogenesis (Figure 3A). Chmp proteins bind to permeabilized membranes following endosomal Ca^{2+} leakage into the cytosol, implicating the ESCRT III complex in membrane repair processes.^{9, 30} Therefore, we chose Chmp1b as a marker of endosomal membrane repair and as a probe for relatively small membrane disruptions.⁹ Upon organelle damage, luminal β -galactosides become exposed to cytosolic components (this presumably requires large membrane disruptions). Cytosolic Galectin-3 (Gal3) binds to these exposed β -galactosides, thereby initiating autophagy.³¹

Recruitment of Gal3 to endosomes is therefore a marker for the degradation of these organelles. Finally, the transcription factor EB (TFEB) regulates the induction of lysosomal biogenesis and autophagy.^{32, 33} TFEB activation involves endosomal Ca^{2+} efflux, activation of the phosphatase calcineurin, dephosphorylation of TFEB, and subsequent translocation of the transcription factor to the nucleus.³⁴ Overall, the endosomal recruitment of Chmp1b and Gal3 and the nuclear accumulation of TFEB upon cell treatment with delivery protocols were monitored by fluorescence microscopy (Figure 3B). The average number of Chmp1b and Gal3 puncta per cell was counted. In addition, because puncta can potentially coalesce to form large aggregates, the total number of pixels from puncta was quantified.

The number of Chmp1b puncta after Lipofectamine, dfTAT and TMR-TAT treatments was similar to that detected in untreated cells (Figure 3B). Similarly, the cytosolic distribution of Gal3 was maintained for these conditions, with only a moderate increase in puncta for Lipofectamine. In contrast, LLOME, UNC7938, dfTAT/UNC7938, and TMR-TAT + hv trigger the recruitment of Chmp1b and Gal3 to damaged membranes. These results were reproduced with Chmp4b and Gal8, additional markers of membrane repair and autophagy (Figure S6). Both Chmp1b and Gal3 colocalized with LAMP1, confirming that both proteins are recruited to damaged late endosomes/lysosomes (Figure 3B). The Chmp1b and Gal3 puncta are distinct after 10 min of LLOME treatment but tend to colocalize after 1h. This suggests an overlap between membrane repair and autophagy pathways, as previously suggested.³⁵ Strikingly, only LLOME and dfTAT/UNC7938 triggered the nuclear translocation of TFEB (bafilomycin being a positive control).

CPP-mediated disruption of endocytic uptake and endosomal degradation

If delivery agents disrupt the membrane of endosomes, one can envision how this may then alter the trafficking of these organelles, and as a result, alter the endocytic pathway as a whole. Based on this idea, we next examined how delivery agents impacted endocytic uptake, an initial step in the maturation of the pathway. In order to quantify endocytic uptake, cells were treated with transferrin (T), the cholera toxin (CT), and histone H1, each protein labeled with Alexa Fluor 488 (AF488). Our results indicated that these probes likely use multiple endocytic uptake mechanisms under the conditions used (Figure S7). We therefore use them in this study as overlapping yet distinct probes to survey the overall effect of CPPs on endocytic uptake. Cells were incubated with probes after treatment with delivery agents or vehicle. Localization of the probes within endosomes was confirmed by microscopy and colocalization with endosomal markers. Uptake was quantified by measuring the AF488 fluorescence intensity associated with each cell population by flow cytometry. In particular, the AF488 intensities that correspond to 50% population thresholds were used for comparison purposes (Figure 4A). With the exception of Lipofectamine, all reagents led to an inhibition of transferrin uptake in both Neuro-2a and HeLa cell lines. In contrast, the uptake of the cholera toxin was either unaffected or increased by most reagents. One exception is the cocktail dfTAT/UNC7938, which, surprisingly, caused an enhancement of uptake in one cell line, but a reduction in another. The uptake of H1 was stimulated by LLOME and TMR-TAT. In contrast, it is inhibited by dfTAT and dfTAT/UNC7938, the latter condition having the most severe effect. Interestingly, UNC7938 alone had a minimal impact on cellular uptake.

Endolysosomal proteolytic degradation is a characteristic function of the endocytic pathway. To assess how it is impacted by the permeation of endosomes, cells were either pretreated with vehicle (L-15) or with dfTAT for one hour (Figure 4B). Cells were subsequently incubated with AF488-H1 and the presence of fluorescent degradation products in cell lysates was detected and quantified by SDS-PAGE. Fluorescence microscopy was used to establish that AF448-H1 is localized within endosomes during these experiments. Degradation may therefore originate from the extracellular milieu, prior to internalization, or from the endocytic pathway, after uptake. In cells treated with vehicle, approximately half of internalized H1 was degraded at the 2h time point. In contrast, the 50% degradation time point is reached at approximately 3.5h when cells are preincubated with dfTAT. Hence, dfTAT causes a delay in degradation kinetics but cells remain overall capable of degrading endocytosed proteins. This delay in degradation kinetics may be caused by a loss of endosomal proteases to the cytosol during dfTAT-mediated leakage. Additionally, dfTAT may render these pH-dependent proteases less active by possibly increasing the pH of endosomes (as noted in Figure S3).

In principle, the high membrane permeation activity of dfTAT and dfTAT/UNC7938 may deleteriously impact the endolysosomal network by changing the number of endocytic organelles present in a cell. To address this question, cells were examined by transmission electron microscopy. Endocytic organelles were counted as either multivesicular organelles (MV, e.g. early endosomes, late endosomes), endolysosomes (EL), or lysosomes (L) based on their distinct morphology.³⁶ In both dfTAT and dfTAT/UNC7938 treated cells, the number of multivesicular organelles decreased while the number of endolysosomes increased (Figure 4C). These changes were approximately inversely proportional, indicating a possible conversion of multivesicular endosomes into endolysosomes upon cell penetration. Notably, dfTAT-treated cells were otherwise indistinguishable from untreated cells. In contrast, cells treated with dfTAT/UNC7938 displayed dense clusters of MV, EL, and L (Figure 4D).

Cellular recovery after endosomal escape and delivery.

Once organelles are either repaired or targeted for degradation, Chmp1b, Gal3, and TFEB eventually return to their basal cytosolic distribution.^{9, 30, 37} Interested in establishing how quickly cells recover from a delivery protocol, we first chose to measure how rapidly Chmp1b and Gal3 puncta disappear from treated cells and how rapidly TFEB returns to a cytoplasmic localization (Figure 5A–C). Both HeLa and Neuro-2a cells were treated with dfTAT/UNC7938, the delivery protocol identified as the most disruptive in our previous experiments. LLOME was used as control when possible (LLOME did not trigger Chmp1b and Gal3 recruitment in Neuro-2a). Gal3 puncta were detected in HeLa cells for up to 18h while Chmp1b puncta were at basal levels in approximately 3h. Gal3 recovery was noticeably more rapid in Neuro-2a than in HeLa. In contrast, Chmp1b recovery was comparatively delayed in Neuro-2a. The rates of recovery between LLOME and dfTAT/UNC7938 were similar, indicating that this process is relatively independent of the agent causing membrane damage. In the case of TFEB, the percentage of cells displaying a nuclear localization of the transcription factor first increased over time for LLOME while being already at its maximum for dfTAT/UNC7938 immediately after incubation. Notably,

nuclear accumulation was steady or rising for 3h in both cases, indicative of a sustained signaling of membrane damage and activation of TFEB. Cells then returned to a cytoplasmic TFEB distribution after an additional 9h. As with Gal3 and Chmp1b, the rate of recovery was overall similar between LLOME and dfTAT/UNC7938, and slower in HeLa cells than in Neuro-2a. Of note, the recovery rates of Gal3 and TFEB were consistently similar, indicating a possible connection in how these responses are orchestrated.

We next monitored the recovery kinetics of endocytic uptake of H1 and LysoTracker staining of cells over time after dfTAT treatment (Figure 5D and E). In these experiments, cells were treated as in Figures S2 and 4. However, in this case, AF488-H1 or LysoTracker were added at increasing intervals after dfTAT treatment and cell washing. As observed before by flow cytometry, the uptake of H1 after dfTAT treatment was decreased by 60% when compared to untreated cells. Endocytic uptake increases progressively overtime, returning to normal levels in less than 5h. In comparison, the recovery of LysoTracker staining was substantially longer, requiring 8h for LLOME, and 12 to 24h for dfTAT in Neuro-2a and HeLa, respectively.

Discussion

A necessary step in cellular delivery applications is the translocation of macromolecules across membranes. Our starting assumption was that, the higher the efficiency of translocation, the higher the membrane damage would be. The basic premise for this assumption lies in the fact that the translocation of macromolecules across a membrane barrier presumably requires a substantial disruption in the integrity of a bilayer. In particular, a reagent like dfTAT is capable of permeating the membrane of late endosomes very efficiently. It is efficient because the majority of macromolecules (>90%) initially entrapped in these organelles can reach the cytosol.²¹ Membrane permeation also appears to accommodate the translocation of large species, including nanoparticles that have a diameter of 50–100 nm.²⁷ We can therefore imagine that dfTAT generates large pores or tears in the membrane and that these membrane disruptions should be detected by repair or degradation pathway. However, based on our results, it is not the case. In particular, dfTAT did not induce the recruitment of Chmp1b or Gal3, nor did it cause the activation of TFEB. Conversely, UNC7938, a reagent that yields endosomal escape with an efficiency below the detection level of our assays, causes Chmp1b and Gal3 responses similar to LLOME, a compound that permeabilizes lysosomes. Moreover, the combination of dfTAT/UNC7938 shows the high levels of endosomal permeation achieved with dfTAT, but displays the Chmp1b and Gal3 responses obtained with UNC7938 alone. These results indicate that, surprisingly, there is no correlation between endosomal escape and membrane repair or clearance responses (Figure 6). In turn, these results establish that high levels of endosomal membrane permeation and cytosolic release can be achieved without necessarily inducing membrane repair, organelle autophagy, or lysosomal biogenesis.

In the absence of precise molecular details on how membrane permeation takes place, it is difficult to interpret how large macromolecular cargo can exit endosomes with the help of dfTAT without eliciting Chmp1b/Gal3 responses. A possible explanation for this phenomenon may relate to the rate at which permeation take place. For instance, as shown in

Figure S3, dFTAT enters the cytosol of cells progressively over a period of 1h. This rate is in part consistent with the time that it may take for the CPP to reach late endosomes. Once in these organelles, it is however possible that dFTAT permeates membranes in transient bursts. Transient membrane leakage would let a small amount of cargo enter the cytosolic space at any given time and it may take several minutes before endosomes lose their content. Concomitantly, calcium could exit endosomes during these short permeation bursts. However, because of the presence of calcium exporters in the cell, the local influx of calcium could be rapidly dissipated before any downstream membrane damage responses are initiated. In contrast, UNC7938 could cause a sustained release of calcium. In particular, given that we were not able to detect cytosolic influx of calcium in neither the dFTAT or UNC7938 conditions, the concentration of calcium released by UNC7938 may not be higher than in the case of dFTAT. However, the continuous release of this ion could be sufficient to initiate calcium-dependent responses. Additionally, it is important to note the sites of membrane leakage attributed to dFTAT are late endosomes.³⁸ These organelles contain intraluminal vesicles rich in the lipid bis(monoacylglycerol) phosphate (BMP) and BMP-containing bilayers are prone to the leakage induced by TAT-like CPPs.^{38, 39} It is therefore conceivable that membrane translocation events involve these vesicles. Macromolecular cargos delivered by dFTAT could for instance enter the lumen of these vesicles and reach the cytosol after back fusion of these vesicles to the limiting membrane of late endosomes. The bilayer permeation would however take place inside the organelle, hidden from membrane repair and degradation machinery contained in the cytoplasm. Alternatively, Pei and co-workers have proposed a vesicle budding and collapse mechanism for the endosomal escape of arginine-rich peptides.⁴⁰ In this model, CPPs induce the formation of vesicles that bud from endosomes. These vesicles subsequently rupture and release their contents into the cytosol. Here again, it is possible that this process could evade membrane damage responses.

Regardless of the mechanistic reasons underlying why UNC7938 triggers membrane damage responses while dFTAT does not, it is clear that our results point to the fact that these delivery agents may be best adapted for different applications. For instance, we have already shown that dFTAT-induced endosomal permeation leads to a minor response in gene expression. Specifically, out of 47,000 mRNA transcript surveyed, dFTAT causes the dysregulation of 11 genes immediately following the delivery protocol and only 2 genes 24h after.²¹ In contrast, Lipofectamine induces the dysregulation of more than 1000 genes.⁴¹ dFTAT does affect LysoTracker staining, endocytic uptake and endosomal protein degradation. However, the relatively weak membrane damage responses detected with this reagent further suggest that dFTAT-mediated delivery is relatively benign to the cells. It may therefore be adequate for cell biology applications that involve investigating the function of a delivered cargo. This is because such studies could be performed in a cellular background that is not dramatically different from that of untreated cells. In contrast, the combination of dFTAT/UNC7938 allows successful delivery for cargos and culture conditions that do not work with dFTAT alone.²² This cocktail clearly induces more pronounced membrane damage responses. Based on the TFEB response and the nuclear retention of this transcription factor, it presumably also leads to a gene-expression response.³³ This delivery protocol can therefore change the characteristics of a cell and using this approach may consequently be less amenable to cell biology studies. Notably, the membrane-damage responses detected

dissipate and cells recover without loss of viability. We therefore propose that this delivery cocktail may be adequate for applications that are compatible with cell recovery, such as gene editing and cell reprogramming. It is important to note that our results however apply to delivery protocols using delivery reagents and payloads that are not covalently linked. It is possible that attaching large cargos to the CPPs used in this study may change their interactions with membranes and subsequently modulate membrane damage response pathways in different ways. Further work is needed to establish this relationship.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CPP-based cytosolic delivery approaches and endosome-disrupting reagents used in this study.

Figure 2.

Reagent-mediated leakage of fluorescent probes trapped in endosomes. SNAP-Surface 488, FI-k5 and AF488-H1 were incubated with delivery reagents for 1h and their cytosolic egress was quantified by counting the number of cells displaying nuclear or nucleolar staining by the probes. For all experiments, the data provided are the mean of triplicate experiments (approximately 500 cells quantified per condition for each experiment).

Figure 4.

Impact of CPPs on endocytic uptake and endosomal degradation post incubation. **A)** Cellular uptake of endocytic probes after treatment with delivery agents. Cells were treated with CPPs for their respective incubation times, washed, and incubated with AF488-labelled probes for endocytic uptake, namely transferrin (T), cholera toxin subunit B (CT), and Histone H1 (H1). The uptake of each probe was quantified by flow cytometry. Four representative flow cytometry traces of the uptake of AF488-H1 are shown. The AF488 intensities that corresponded to a 50% threshold (dotted lines) was determined for each condition. The fold change in uptake for each condition as compared to the vehicle/probe control is shown. The values reported represent the mean of biological duplicates. The standard deviations (not shown) are 0.2 or less. **(B)** Impact of dFTAT on endosomal proteolysis. The degradation of the probe AF488-H1 endocytosed by cells was monitored over time by quantifying the formation of fluorescent protein fragments by SDS-PAGE. The degradation rate of AF488-H1 endocytosed was compared for cells preincubated for 1h with either vehicle or dFTAT. Each plotted value represents the mean fluorescence intensity of intact or degraded AF488-H1 from biological duplicates. Fluorescence microscopy was used to compare the localization of H1 incubated with cells during or after incubation with dFTAT. Images are pseudocolored green for AF488-H1 and magenta for Hoechst (scale 10 μm). **(C)** Transmission electron microscopy imaging of cells treated with dFTAT or dFTAT/UNC7938. The number of multivesicular bodies/late endosomes, endolysosomes, and lysosomes were

counted from the cross sections of 5 cells. (D) Representative TEM images of cells treated with vehicle (left) and dfTAT/UNC7938-treated (right). Organelles are designated as multivesicular bodies/late endosomes (MV), endolysosomes (EL), lysosomes (L) based on their morphologies.

Figure 5.

Recovery kinetics of membrane damage probes. (A) and (B) The number Gal3 or Chmp1b positive puncta per cell was quantified over time after LLOME or dfTAT/UNC7938 treatment. The data are represented as box and whiskers plots. (C) Percentage of cells displaying a nuclear localization of TFEB post LLOME or dfTAT/UNC7938 treatment. (D) Cellular recovery of AF488-H1 uptake after treatment with dfTAT for 1h. (E) Recovery of LysoTracker Green staining in cells were treated with LLOME or dfTAT. For all experiments, the data are obtained from biological triplicates.