

RESEARCH ARTICLE

Shiga toxin stimulates clathrin-independent endocytosis of the VAMP2, VAMP3 and VAMP8 SNARE proteins

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ABSTRACT

Endocytosis is an essential cellular process that is often hijacked by pathogens and pathogenic products. Endocytic processes can be classified into two broad categories, those that are dependent on clathrin and those that are not. The SNARE proteins VAMP2, VAMP3 and VAMP8 are internalized in a clathrin-dependent manner. However, the full scope of their endocytic behavior has not yet been elucidated. Here, we found that VAMP2, VAMP3 and VAMP8 are localized on plasma membrane invaginations and very early uptake structures that are induced by the bacterial Shiga toxin, which enters cells by clathrin-independent endocytosis. We show that toxin trafficking into cells and cell intoxication rely on these SNARE proteins. Of note, the cellular uptake of VAMP3 is increased in the presence of Shiga toxin, even when clathrin-dependent endocytosis is blocked. We therefore conclude that VAMP2, VAMP3 and VAMP8 are removed from the plasma membrane by non-clathrin-mediated pathways, in addition to by clathrin-dependent uptake. Moreover, our study identifies these SNARE proteins as the first transmembrane trafficking factors that functionally associate at the plasma membrane with the toxin-driven clathrin-independent invaginations during the uptake process.

KEY WORDS: Clathrin-independent endocytosis, SNARE, Shiga toxin, VAMP2, VAMP3, VAMP8

INTRODUCTION

Endocytosis has emerged as an essential cellular process, and a focus of research in cell biology. Nutrients and signaling molecules are internalized by endocytosis from the cellular environment, and plasma membrane components are turned over. Endocytic uptake has fundamental implications on diverse cellular functions such as adhesion and migration, polarity and division, and growth and differentiation (Blouin and Lamaze, 2013; Doherty and McMahon, 2009). Since its discovery over 50 years ago, clathrin-mediated endocytosis has been by far the most widely studied endocytic route (Kirchhausen et al., 2014; McMahon and Boucrot, 2011). Clathrin triskelia polymerize at the plasma membrane and form a polygonal lattice that drives membrane bending and the biogenesis of endocytic pits. Adaptors like AP2 and CALM interact with clathrin, and allow the recruitment of transmembrane cargo proteins to endocytic pits. However, data have started to accumulate since the 1980s on the

existence of endocytic processes that operate independently of clathrin (reviewed in Blouin and Lamaze, 2013; Doherty and McMahon, 2009; Mayor et al., 2014; Sandvig et al., 2011), including the cellular uptake of the bacterial Shiga toxin (STx) (Renard et al., 2015; Römer et al., 2007).

Shiga toxin is composed of two subunits, A and B (Johannes and Römer, 2010). The catalytic A-subunit modifies ribosomal RNA in the cytosol of target cells, leading to protein biosynthesis inhibition. To reach the cytosol, the A-subunit non-covalently interacts with the homopentameric B-subunit (STxB). STxB binds to the cellular toxin receptor, the glycosphingolipid Gb3, and then shuttles the holotoxin through the retrograde route from the plasma membrane to the endoplasmic reticulum (ER), via early endosomes and the Golgi complex. From the lumen of the ER, the A-subunit is translocated to the cytosol to modify its molecular target (Lord et al., 2005).

A model has been proposed to explain how endocytic pits are built in the process of clathrin-independent uptake of Shiga toxin (Johannes et al., 2014). According to this model, STxB reorganizes membrane lipids under toxin molecules (Solovyeva et al., 2015), thus endowing the corresponding membrane patch with curvature-active properties that lead to the narrow bending of the plasma membrane without the involvement of the clathrin machinery (Römer et al., 2007). Cytosolic machinery, such as actin (Römer et al., 2010), dynamin (Römer et al., 2007) and endophilin-A2 (Renard et al., 2015), is then recruited to these invaginations for their processing into cells by scission. Inside cells, newly formed STxB-containing endocytic carriers have to fuse with endosomal compartments for further trafficking into the retrograde route. The molecules that enable the targeting of STxB-containing carriers to and their fusion with endosomes still needed to be identified.

Proteins of the SNARE family are key constituents of the intracellular membrane fusion machinery (Sudhof and Rothman, 2009). This fusion activity requires the formation of trans-SNARE complexes composed of vesicle SNAREs (v-SNAREs, also termed R-SNAREs) on vesicle membranes, and target SNAREs (t-SNAREs, also termed Q-SNAREs) on target compartment membranes. Several studies have identified a complement of v-SNARE proteins of the vesicle-associated membrane protein (VAMP) family that function in trafficking processes from or to the plasma membrane. In adipocytes, VAMP2, VAMP3 and VAMP8 promote the fusion of glucose transporter type 4 (GLUT4, also known as SLC2A4) storage vesicles with the plasma membrane (Stockli et al., 2011; Zhao et al., 2009). In mast cells, VAMP7 and VAMP8 are required for the degranulation process, allowing the release of proinflammatory mediators (Sander et al., 2008). VAMP3 has been described to play a role in the recycling of transferrin receptor, for iron uptake into cells (Galli et al., 1994), and of β 1 integrin, for the regulation of cell adhesion and migration (Proux-Gillardeaux et al., 2005). Several other studies have shown that

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SNARE complexes involving VAMP2, VAMP3 or VAMP8, and SNAP23 and syntaxin-4 mediate the fusion of secretory vesicles with plasma membrane (Kawanishi et al., 2000; Sander et al., 2008; Wang et al., 2010). Moreover, VAMP3 and VAMP4 have been found in SNARE complexes that mediate retrograde transport between endosomal compartments and the trans-Golgi network (TGN) (Ganley et al., 2008; Johannes and Wunder, 2011; Mallard et al., 2002; Tran et al., 2007). Recently, several studies have demonstrated that VAMP2, VAMP3 and VAMP8 interact with the clathrin adaptor CALM (also known as PICALM) for their retrieval from the plasma membrane by clathrin-dependent endocytosis (Harel et al., 2008; Koo et al., 2011a,b; Miller et al., 2011). However, virtually no data exist about the function of VAMP proteins in clathrin-independent endocytic processes, such as the Shiga toxin uptake pathway.

In this study, we screened an expression library of all members of the VAMP family for their localization to STxB-induced plasma membrane invaginations. Among the hits, only VAMP2, VAMP3 and VAMP8 had a functional role in retrograde transport of STxB to perinuclear Golgi membranes. Live-cell imaging showed that STxB was taken up within a few seconds into VAMP2-, VAMP3- and VAMP8-positive endocytic carriers. Interestingly, the endocytosis of VAMP3 was increased in the presence of STxB, even when clathrin-dependent endocytosis was blocked by depletion of the clathrin adaptor protein CALM. Based on these results, we propose that VAMP2, VAMP3 and VAMP8 are also internalized by clathrin-independent endocytosis, and that these SNARE proteins constitute the first transmembrane machinery that functionally associates with the Shiga-toxin-driven uptake pathway at the plasma membrane.

RESULTS

VAMP2, VAMP3 and VAMP8 function in STxB trafficking into cells

To be functional, STxB-induced endocytic carriers need to contain targeting information and fusion machinery. To identify SNARE proteins that enable STxB trafficking into cells, we performed an expression screen on HeLa cells, as described previously (Renard et al., 2015). Five out of seven GFP-tagged VAMP proteins (Fig. 1A, green overlay; supplementary material Fig. S1A,B) and three out of six syntaxins (supplementary material Fig. S2A) were found to localize on STxB-induced plasma membrane invaginations. Screening results obtained under ATP depletion conditions were confirmed using dynamin depletion as an alternative means to stabilize STxB-induced tubules (supplementary material Fig. S1A, right panel 'siDYN').

For the current study, we focused our interest on VAMP proteins. Primary hits (Fig. 1A, green overlay) were submitted to a secondary screen for functional validation in which the corresponding VAMP proteins were depleted using small interfering RNAs (siRNAs), followed by retrograde transport analysis using the sulfation assay (Amessou et al., 2006). In this assay, retrograde transport from the plasma membrane to the TGN is measured using a STxB variant with tandem protein sulfation sites, termed STxB-Sulf₂. Following arrival in the TGN, this protein is sulfated by resident tyrosylsulfotransferases, using radioactive [³⁵S]sulfate from the incubation medium. Radiolabeled STxB-Sulf₂ is then immunoprecipitated for quantification by autoradiography. siRNA-mediated depletion of VAMP1 or of VAMP5 did not have a significant effect in this assay (Fig. 1B, gray bars), whereas single depletion of VAMP2, VAMP3 or VAMP8 each reduced Golgi arrival of STxB-Sulf₂ to 58.9% (VAMP2, sequence #5), 62.6% (VAMP2, sequence #4), 61.9% (VAMP3, sequence #2),

52.5% (VAMP3, sequence #5), 59.7% (VAMP8, sequence #5) or 59.3% (VAMP8, sequence #4) of AllStars negative control siRNA-transfected cells (Fig. 1B). Combined depletion of VAMP2, VAMP3 and VAMP8 (hereafter VAMP2/3/8-depleted cells) led to a stronger reduction to 38.2% (pool 1 with VAMP2 #5, VAMP3 #2 and VAMP8 #5 sequences) or 47.9% (pool 2 with VAMP2 #4, VAMP3 #5 and VAMP8 #4 sequences) (Fig. 1B, green bars). Syntaxin-16 knockdown was used as a benchmark treatment (Fig. 1B). The efficiency of the different siRNA treatments was validated by western blotting (supplementary material Fig. S2B). The reduced arrival of STxB to Golgi membranes was also confirmed when VAMP2/3/8-depleted cells were analyzed by immunofluorescence after incubation for 45 min at 37°C with fluorescently labeled STxB (Fig. 1C). Under these conditions, a sizable fraction of STxB remained localized in peripheral endosomal structures.

Protein biosynthesis inhibition is a well-established and extremely sensitive measure of Shiga toxin arrival in the cytosol, following trafficking via the retrograde route (Johannes and Romer, 2010). VAMP2/3/8-depleted HeLa cells required 3.6-fold (± 0.3 , mean $\pm$ s.e.m.) more Shiga-like toxin-1 (note that Shiga-like toxin-1 and Shiga toxin are almost identical with one conserved amino acid difference) than control cells to reach the same level of protein biosynthesis inhibition (Fig. 1D), documenting that these SNARE proteins were indeed required for efficient toxin uptake.

To assess the binding capacity of STxB on VAMP2/3/8-depleted HeLa cells, these were incubated on ice with Alexa-Fluor-488-labeled STxB, and analyzed by flow cytometry. When compared to mock-depleted control cells, the same plasma membrane binding capacity was observed (supplementary material Fig. S2C,E,F). This demonstrated that the decreased STxB sulfation or protein biosynthesis levels in VAMP2/3/8-depleted cells were not due to reduced amounts of cell-associated STxB. In contrast, the binding capacity of transferrin was significantly reduced in VAMP2/3/8-depleted HeLa cells (supplementary material Fig. S2D–F), as described previously for cells in which VAMP3 was cleaved by tetanus toxin (Galli et al., 1994).

VAMP2, VAMP3 and VAMP8 localization on STxB-induced plasma membrane invaginations was confirmed using cell lines that expressed HA-tagged versions of these SNAREs at levels below those of the endogenous proteins (Miller et al., 2011), thereby minimizing the risk of mislocalization artifacts. Control cells showed labeling of anti-HA antibodies on STxB-induced tubules that were stabilized by ATP depletion (supplementary material Fig. S3A, see magnifications and arrows). This phenotype was enhanced upon depletion of CALM, where HA-tagged VAMP2, VAMP3 and VAMP8 strongly localized on tubular STxB-containing structures (supplementary material Fig. S3B, see magnifications and arrows). CALM depletion and expression levels of HA-tagged VAMP proteins were verified by western blotting (supplementary material Fig. S3C–E). Note that CALM depletion also induced an ~60–70% decrease of the clathrin heavy chain signal. We confirmed these results with HA-tagged proteins using an antibody against endogenous VAMP3, which also localized to STxB-induced membrane invaginations, especially on CALM-depleted cells (supplementary material Fig. S3F,G). It thereby became apparent that under conditions of decreased uptake via the clathrin route, VAMP2, VAMP3 and VAMP8 preferentially distributed into the Shiga toxin pathway.

Taken together, these data demonstrate that VAMP2, VAMP3 and VAMP8 localize onto STxB-induced membrane invaginations, and that these three v-SNARE proteins are important for efficient retrograde transport of the toxin.

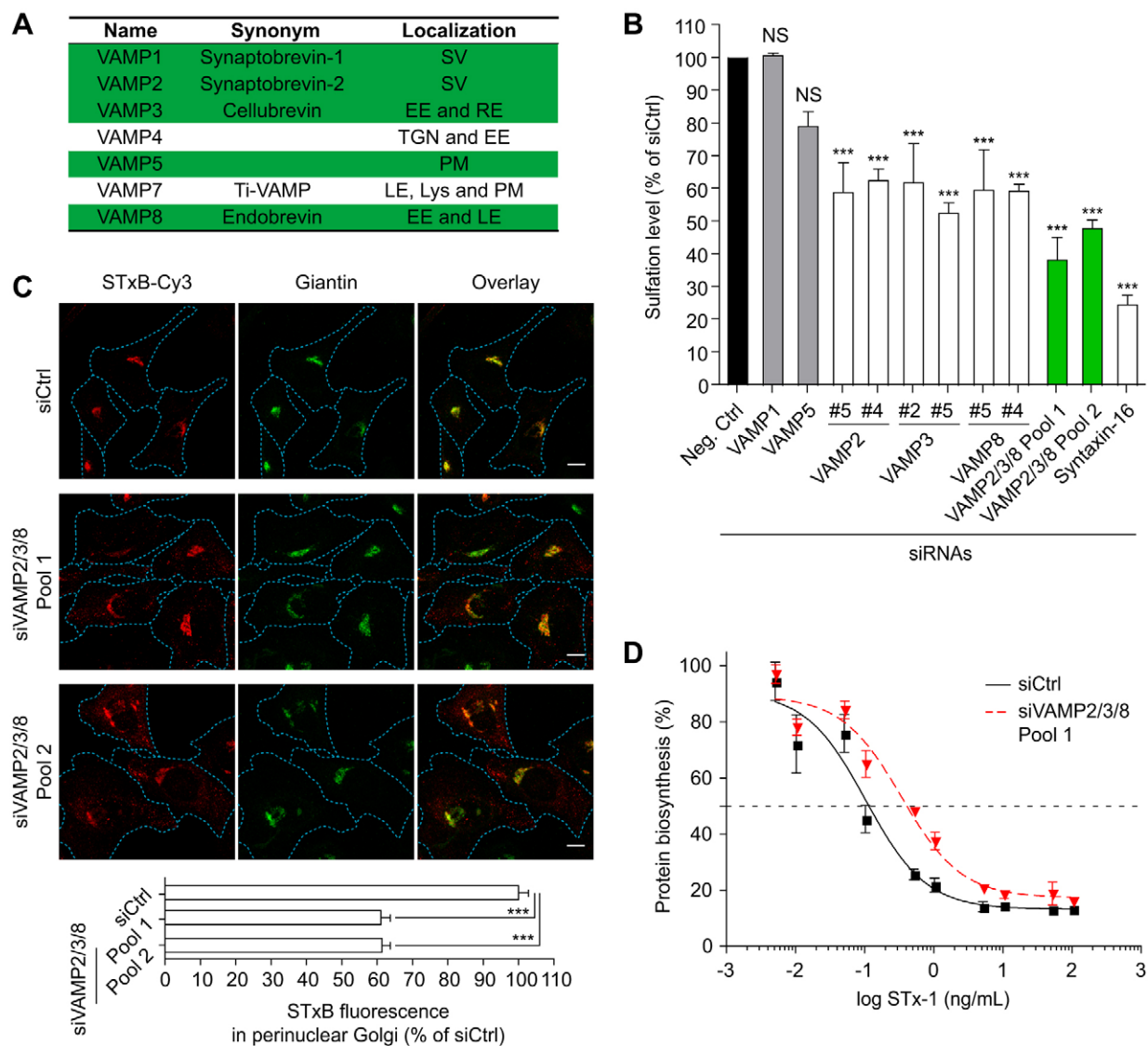


Fig. 1. Visual and functional screening. (A) Table of v-SNAREs that were screened in this study for localization to STxB-induced plasma membrane invaginations. Green overlay indicates positive hits. (B) Secondary functional screening. VAMP proteins that scored positive in the visual screening were depleted using siRNAs, and a sulfation analysis was performed to measure retrograde STxB transport to the Golgi complex. Amongst the VAMPs that were tested, the combined depletion of VAMP2, VAMP3 and VAMP8 had the strongest inhibitory effect (siRNAs pool 1, VAMP2 #5, VAMP3 #2 and VAMP8 #5; siRNAs pool 2, VAMP2 #4, VAMP3 #5 and VAMP8 #5). Depletion of syntaxin-16 was used as a benchmark treatment. $n=4$ independent experiments. NS, non significant. $***P<0.001$ (Bonferroni's multiple comparison test). (C) Immunofluorescence analysis of STxB transport to the perinuclear Golgi on control (siCtrl) and VAMP2/3/8-depleted HeLa cells (siVAMP2/3/8 pools 1 or 2, as in B). Giantin staining (green) was used to label the position of the Golgi. Note the pronounced peripheral STxB–Cy3 staining in the VAMP2/3/8-depleted cells (siVAMP2/3/8). Quantifications represent the percentage of STxB–Cy3 fluorescence signal in the Golgi area (as defined by giantin) versus the whole cell (siCtrl, $n=372$ cells, five independent experiments; siVAMP2/3/8 Pool 1, $n=336$ cells, five independent experiments; siVAMP2/3/8 Pool 2, $n=85$ cells; two independent experiments). $***P<0.001$ (Bonferroni's multiple comparison test). Scale bars: 10 μm . (D) Cell intoxication experiments with STx-1 on control (siCtrl) or VAMP2/3/8-depleted cells (siRNAs pool 1, siVAMP2/3/8). Protein biosynthesis levels were measured with increasing doses of STx-1. A protection factor of $3.6 (\pm 0.3)$ was calculated from three independent experiments. Mean $\pm$ s.e.m. are shown.

STxB stimulates clathrin-independent endocytosis of VAMP2, VAMP3 and VAMP8

To study the localization of VAMP2, VAMP3 and VAMP8 on STxB-containing endocytic carriers shortly after uptake, we incubated Cy5-labeled STxB with HeLa cells that transiently expressed GFP-tagged VAMP2, VAMP3 or VAMP8, and observed these cells by spinning disk microscopy. Between 30–60 s after STxB binding to the cell surface, carriers positive for both STxB–Cy5 and GFP-tagged VAMP2, VAMP3 or VAMP8 started to appear close to cell periphery (Fig. 2, white arrows; supplementary material Movies 1–3). These data demonstrate that these three v-SNAREs are found on very early STxB uptake carriers.

Next, we asked whether the efficacy of endocytic uptake of these SNAREs could be modulated by STxB. For this, we set up an anti-HA antibody uptake assay on the cell line stably expressing HA-tagged VAMP3, exploiting the fact that the HA tag is facing the extracellular medium. After binding on ice of Alexa-Fluor-488-labeled anti-HA antibody to cells that concomitantly had been incubated or not with Cy3-labeled STxB, these were shifted for 5 or 15 min to 37°C. Average intensities of internalized anti-HA–Alexa-Fluor-488 antibody per cell were quantified from confocal images, both in control conditions and for cells in which CALM was depleted using specific siRNAs. Under CALM depletion conditions, it has previously been described that HA–VAMP3 accumulates at the

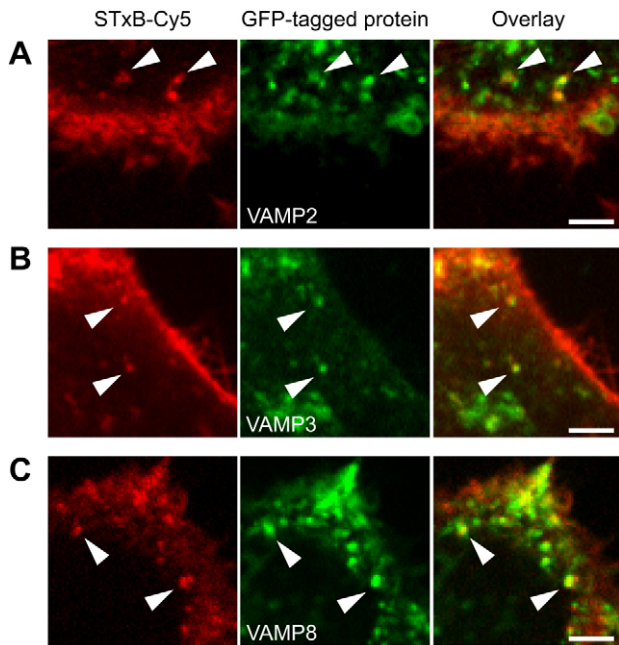


Fig. 2. Live-cell imaging of STxB endocytosis. HeLa cells were transiently transfected with the indicated GFP-tagged VAMP constructs, continuously incubated with 200 nM Cy5-labeled STxB, and imaged by spinning disk microscopy. After 30–60 s, uptake carriers positive for both STxB and the different GFP-tagged VAMPs (indicated by white arrows) were detected inside cells. See supplementary material Movies 1, 2 and 3 for the complete image series. Scale bars: 2 μ m.

plasma membrane, and that its endocytic uptake is very strongly inhibited (Miller et al., 2011). Here, we found that in the presence of STxB, a significant fraction of Alexa-Fluor-488-labeled antibody was internalized after a 15-min incubation period at 37°C (Fig. 3A, +STxB), whereas very little internalization of antibody was observed in the absence of STxB (Fig. 3A, –STxB). Indeed, although very few vesicles positive for anti-HA antibody were observed in the absence of STxB (Fig. 3B, upper panel), their number was clearly increased in the presence of STxB (Fig. 3B, lower panel). This stimulatory effect was dose-dependent. Significant uptake was measured with doses as little as 40 nM of STxB, and a plateau was reached at 200 nM of STxB (supplementary material Fig. S4A). STxB thus stimulated VAMP3 uptake in a dose-dependent and clathrin-independent manner.

Of note, the STxB-dependent VAMP3 uptake stimulation phenotype was not as such linked to the CALM depletion condition. Even on AllStars negative control siRNA-transfected cells, the internalization of VAMP3 was increased in the presence of STxB (supplementary material Fig. S4B). Taken together, these data document that VAMP3 was also internalized into cells in a clathrin-independent manner, in tight association with the Shiga toxin entry mechanism.

Sites of STxB accumulation in cells depleted for VAMP2, VAMP3 and VAMP8

In Fig. 1C, we documented an increased accumulation of STxB in peripheral structures upon depletion of VAMP2, VAMP3 and VAMP8, indicating that either STxB arrival in these structures was delayed due to inefficient fusion, and/or that STxB exit was retarded due to inefficient trafficking to TGN membranes. To identify the endosomal nature of these compartments, we colocalized the toxin with different markers: Rab11 and transferrin receptor (TfR) for early and recycling endosomes (Fig. 4A,B), Rab5 for early endosomes (Fig. 4C), and LAMP1 for late endosomes and lysosomes (Fig. 4D). The percentage of STxB-positive

structures that colocalized with these markers was determined, using an object-based method (see Materials and Methods). The efficiency of VAMP2, VAMP3 and VAMP8 depletion was assessed by western blotting and was similar for all conditions and all subsequent experiments (supplementary material Fig. S4C). We observed that in VAMP2/3/8-depleted cells, STxB mainly colocalized with early and recycling endosomes (Fig. 4E). In contrast, very little overlap was found with late endosomal or lysosomal compartments (Fig. 4E), as expected based on previous findings (Mallard et al., 1998). Taken together, these data show that STxB is blocked or delayed in pre- and early endosomal compartments under VAMP2, VAMP3 and VAMP8 depletion conditions.

Effects of VAMP2, VAMP3 and VAMP8 depletion on other trafficking routes

Given that STxB transport to the TGN was inhibited in VAMP2/3/8-depleted cells, we tested whether other retrograde trafficking routes were also affected. Cation-independent mannose-6-phosphate receptor (CI-MPR, also known as IGF2R) and TGN46 (also known as TGOLN2) are two well-characterized retrograde cargo proteins (Johannes and Popoff, 2008). We observed that the concomitant depletion of VAMP2, VAMP3 and VAMP8 did not affect the distribution of both CI-MPR (Fig. 5A) or TGN46 (Fig. 5B). Giantin (also known as GOLGB1), a peripheral Golgi protein, was used as a reference point.

Epidermal growth factor (EGF) receptor (EGFR) is internalized by clathrin-dependent and independent endocytosis, depending on EGF concentration (Dikic, 2003). To measure lysosomal degradation following endocytic uptake, EGFR was detected by western blotting at different time points after addition of 100 ng/ml of EGF into the growth medium. Depletion of VAMP2, VAMP3 and VAMP8 did not measurably delay this degradation kinetics (Fig. 6A), demonstrating that trafficking into the late endocytic pathway was not altered under these conditions.

An ELISA-based assay was then used to measure endocytosis and recycling of human diferric transferrin (Tf). Tf uptake was slightly stimulated (Fig. 6B), and its recycling slightly inhibited (Fig. 6C), as expected (Galli et al., 1994). Both effects were likely responsible for the reduced plasma membrane binding capacity of Tf (supplementary material Fig. S2D–F).

Finally, we examined the effect of concomitant depletion of VAMP2, VAMP3 and VAMP8 on the biosynthetic/secretory pathway. We focused on E-cadherin, a well-characterized anterograde transport cargo protein. Using the ‘Retention Using Selective Hooks’ (RUSH) system (Boncompain et al., 2012), E-cadherin was reversibly anchored in the ER through a fusion construct composed of streptavidin-binding protein (SBP) and mCherry. Upon addition of biotin, SBP–mCherry–E-cadherin was released from an ER-resident membrane-bound streptavidin–hook construct. The arrival of SBP–mCherry–E-cadherin at the plasma membrane was monitored by incubating non-permeabilized cells in the presence of an anti-mCherry antibody. At 1 h after the release, the amount of SBP–mCherry–E-cadherin detected at plasma membrane was decreased by 21.4% in VAMP2/3/8-depleted cells (Fig. 7), consistent with previous studies that have ascribed a function in anterograde transport to these SNARE proteins (see Introduction).

DISCUSSION

Shiga toxin has been well characterized for its capacity to induce narrow plasma membrane invaginations as the first step of its clathrin-independent uptake into cells (Johannes et al., 2014; Römer et al., 2007). A number of cytosolic proteins have been shown to

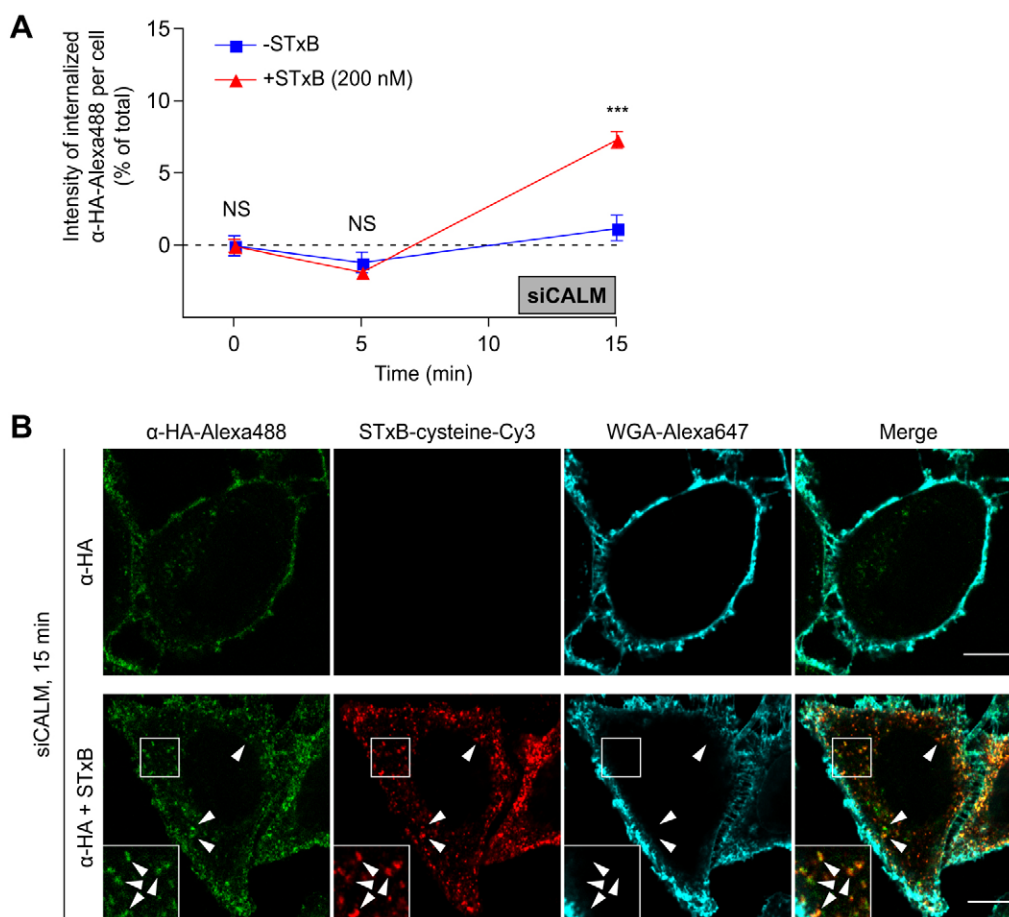


Fig. 3. VAMP3 endocytosis is increased in the presence of STxB. (A) HeLaM cells stably expressing a low level of VAMP3–HA and depleted for CALM (siCALM) were incubated for 45 min on ice with Alexa-Fluor-488-labeled antibodies against HA (α -HA-Alexa488) alone (–STxB, blue), or in the presence of 200 nM STxB/Cys–Cy3 (+STxB, red). Cells were then shifted for 5 or 15 min to 37°C. The mean $\pm$ s.e.m. intensity of internalized anti-HA antibody labeling per cell was determined, and normalized to the 0 min condition. Three independent experiments ($n=359$ to 418 cells per condition). NS, non significant. *** $P<0.001$ (two-way ANOVA with Bonferroni post-tests). Note the significant uptake of VAMP3 in the presence of Shiga toxin. (B) Representative images at 15 min endocytosis for conditions as in A. Note the increased number and intensity of uptake carriers positive for anti-HA antibodies (α -HA-Alexa488) in the presence of Shiga toxin (lower panel, indicated with white arrows) compared to the anti-HA antibody alone incubation conditions (upper panel). Strong colocalization between anti-HA antibody-tagged VAMP3 and STxB was observed (see insets). Scale bars: 10 μ m.

functionally associate with these invaginations (Renard et al., 2015; Römer et al., 2007, 2010; Rydell et al., 2014). Here, we identified the first cellular transmembrane machinery, the v-SNAREs VAMP2, VAMP3 and VAMP8, to localize to Shiga-toxin-induced plasma membrane invaginations and very early endocytic carriers, to be required for efficient retrograde toxin transport to the Golgi complex, and to be necessary for cell intoxication. Interestingly, the endocytosis of VAMP3 was increased in a dose-dependent manner in the presence of Shiga toxin, even when the clathrin adaptor protein CALM was depleted. These data suggest the exciting possibility that these VAMPs are in part internalized by clathrin-independent endocytosis, thereby enabling the trafficking of Shiga-toxin-containing endocytic carriers to intracellular compartments.

VAMP2, VAMP3 and VAMP8 uptake by clathrin-independent endocytosis

Although clathrin-dependent endocytosis is crucial for the retrieval of VAMP2, VAMP3 and VAMP8 from the plasma membrane (Harel et al., 2008; Koo et al., 2011a,b; Miller et al., 2011), it has previously already been suggested that clathrin-independent pathways might also contribute (Xu et al., 2013). Direct evidence for this was however lacking. Here, we show that STxB stimulated the cellular uptake of these v-SNARE proteins, both in unperturbed cells and under CALM depletion conditions. Further investigations will be required to determine whether the intracellular fate of VAMP2, VAMP3 and VAMP8 depends on specific endocytic mechanisms, as has been shown previously for EGFR (Sigismund et al., 2008) and TGF β (Di Guglielmo et al., 2003).

How VAMP2, VAMP3 and VAMP8 are recruited to sites of Shiga toxin internalization also needs to be addressed in future studies. Given that only two to four C-terminal amino acids of these VAMPs are exposed to the extracellular space, it is rather unlikely that recruitment involves a direct interaction with the toxin or any other extracellular driver of clathrin-independent pit construction, such as galectins (Lakshminarayan et al., 2014). The transmembrane domain could contribute to recruitment if it had increased affinity for a specific membrane environment that is likely to be induced by toxin-driven clustering of glycosphingolipid receptor molecules (Pezeshkian et al., 2015; Solovyeva et al., 2015; Watkins et al., 2014). Alternatively, these v-SNAREs could directly or indirectly interact with BAR domain proteins that are recruited to membrane invaginations in clathrin-independent uptake pathways. Recent studies have indeed shown that endophilin-A2 is specifically and functionally associated with Shiga-toxin-induced endocytic carriers (Renard et al., 2015), and also participates in the clathrin-independent uptake of a number of cellular proteins by binding to their cytosolic tails (Boucrot et al., 2015). Finally, a contribution of the actin cytoskeleton to the molecular focusing of VAMP2, VAMP3 and VAMP8 in areas of clathrin-independent endocytosis represents another attractive possibility (Rao and Mayor, 2014).

VAMP2, VAMP3 and VAMP8 function in Shiga toxin uptake

Concomitant depletion of VAMP2, VAMP3 and VAMP8 led to a decreased retrograde trafficking efficiency of STxB to TGN or Golgi membranes, and to a shift of the intoxication curve. Of note, the difference in cell intoxication between control and depletion conditions became visible at low toxin concentrations (between

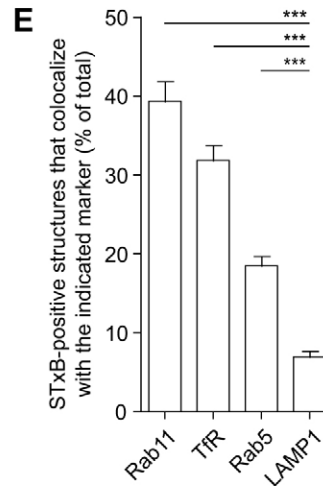
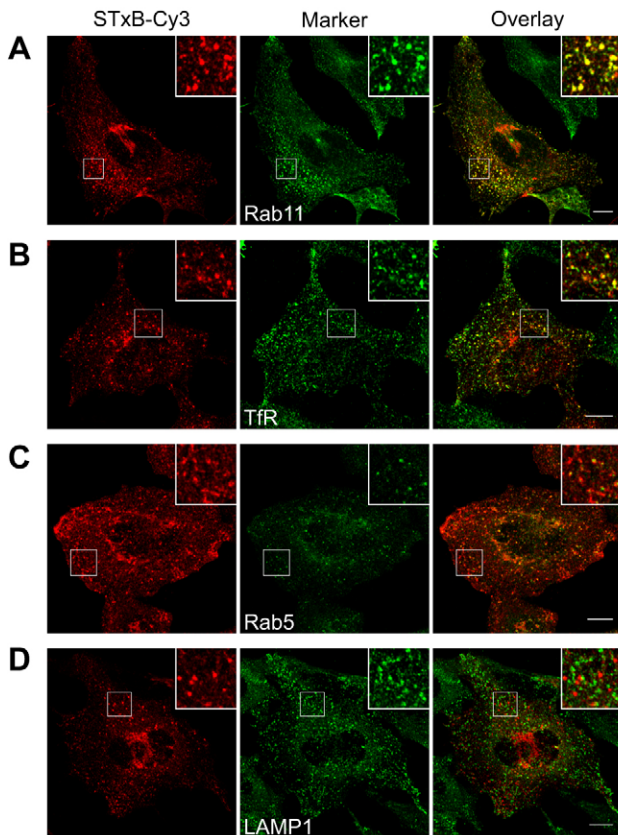


Fig. 4. Endosomal localization of STxB in VAMP2/3/8-depleted cells. (A–D) VAMP2/3/8-depleted HeLa cells (siRNA pool 1) expressing or not expressing Rab11–GFP or Rab5–GFP (green) were incubated on ice with 50 nM STxB–Cy3 (red), and shifted for 45 min to 37°C, fixed, and labeled or not for transferrin receptor (TfR, green) or LAMP1 (green). Scale bars: 10 μ m. (E) STxB-positive structures that colocalized with these markers were quantified as a percentage of the total STxB-positive structures, using an object-based approach. Two independent experiments (Rab11–GFP, $n=24$ cells; TfR, $n=24$ cells; Rab5, $n=19$ cells; LAMP1, $n=26$ cells). *** $P<0.001$ (Bonferroni's multiple comparison test). Note that the high degree of colocalization between STxB and Rab11 or TfR. Means $\pm$ s.e.m. are shown.

0.01 and 10 ng/ml), which one might consider as relevant to the infectious disease condition. It should be pointed out, however, that it is actually not known what the effective toxin concentration is in diseased tissues, largely due to the fact that Shiga toxin is not present as a freely diffusible species in the blood of hemolytic uremic syndrome patients (Brigotti et al., 2011). The toxin appears to associate with chaperoning cells, notably platelets, neutrophils and monocytes (Brigotti et al., 2006; Stahl et al., 2009), from which it would then relocate to target cells by yet unknown mechanisms that possibly involve cell-derived microvesicles (Stahl et al., 2015).

The colocalization of STxB with TfR and Rab11 that was observed in our study upon VAMP2, VAMP3 and VAMP8 depletion at time points where the toxin was mostly in the Golgi under control conditions suggests that its intracellular progression was delayed upstream of early or recycling endosomes, such that these were reached later than usual, and/or that its exit from early or recycling endosomes was delayed such that the toxin stayed longer than usual in these structures. Interestingly, VAMP2 and VAMP3 have been shown to mediate the fusion of plasma-membrane-derived transport carriers with early or recycling endosomes, involving the t-SNARE protein syntaxin-13 (Prekeris et al., 1998). These two VAMPs could thus contribute to early steps of Shiga toxin trafficking into the endosomal system. VAMP3 has also been involved in retrograde toxin trafficking between early or recycling endosomes and the TGN, in complex with syntaxin-16, syntaxin-6 and Vti1a (Mallard et al., 2002). This latter function would account for a post-early or recycling endosomal perturbation. VAMP8 regulates homotypic fusion of early endosomes (Antonin et al., 2000), and could contribute, through this function, to Shiga toxin trafficking.

In conclusion, the current study has identified clathrin-independent endocytosis as an alternative uptake pathway for VAMP2, VAMP3 and VAMP8, and has ascribed functions to these

proteins in the internalization process of Shiga toxin, starting with their localization to Shiga-toxin-induced plasma membrane invaginations and very early clathrin-independent uptake carriers. Single-molecule tracking experiments should allow future studies to determine the exact fraction of these v-SNARE proteins that traffic through the respective endocytic pathways, as well as their intracellular trajectories, which would enable us to assess the function of the pathway by which they are taken up into cells.

MATERIALS AND METHODS

Antibodies and other reagents

The following antibodies were purchased from the indicated suppliers: mouse monoclonal purified anti-HA (Covance, catalog no. MMS-101P, 1:1000 for immunofluorescence and western blotting) and Alexa-Fluor-488-labeled anti-HA (Covance, catalog no. A488-101L, 1:100 for antibody uptake); mouse monoclonal anti- α -tubulin (Sigma, catalog no. T5168, 1:5000 for western blotting); rabbit polyclonal anti-syntaxin-16 (Synaptic Systems, catalog no. 110 163, 1:1000 for western blotting); rabbit monoclonal anti-giantin (Institut Curie, recombinant proteins platform, catalog no. A-R-R#05, 1:100 for immunofluorescence); mouse monoclonal anti-transferrin receptor (BD Pharmingen, catalog no. 555534, 1:100 for immunofluorescence); mouse monoclonal anti-LAMP1 (BD Pharmingen, catalog no. 555798, 1:200 for immunofluorescence); sheep polyclonal anti-TGN46 (Serotec, catalog no. AHP500G, 1:200 for immunofluorescence); mouse monoclonal anti-CI-MPR (Abcam, catalog no. ab2733, 1:200 for immunofluorescence); rabbit monoclonal anti-EGF receptor (Cell Signaling, catalog no. 4267, 1:4000 for western blotting); mouse monoclonal anti-VAMP2 (R&D Systems, catalog no. MAB5136, 1:1000 for western blotting); goat polyclonal anti-VAMP8 (R&D Systems, catalog no. AF5354, 1:1000 for western blotting); rabbit polyclonal anti-mCherry (Institut Curie, recombinant proteins platform, catalog no. A-P-R#13, 1:200 for immunofluorescence); mouse monoclonal anti-clathrin heavy chain (BD Biosciences, catalog no. 610500, 1:5000 for western blotting); goat

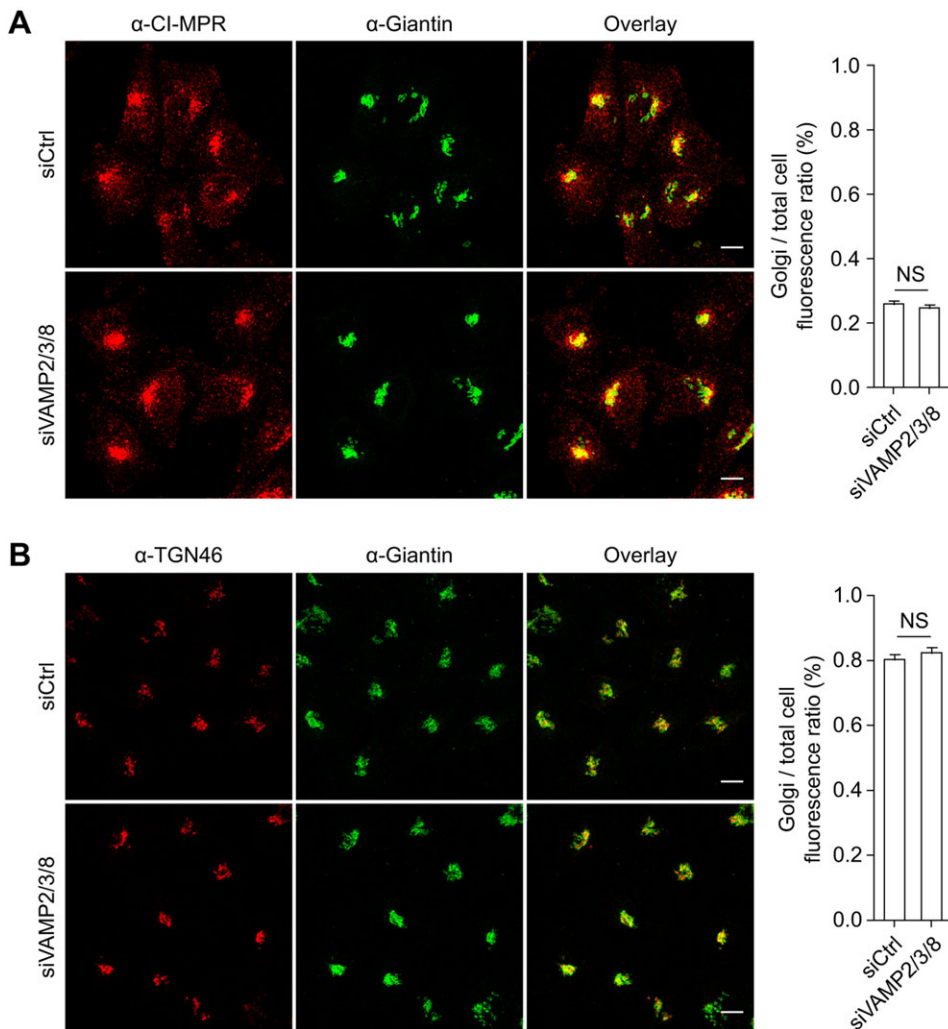


Fig. 5. Depletion of VAMP2, VAMP3 and VAMP8 does not affect CI-MPR or TGN46 localization. Endogenous CI-MPR (A) and TGN46 (B) were detected by immunofluorescence in control (siCtrl) or VAMP2/3/8-depleted (siRNA pool 1, siVAMP2/3/8) HeLa cells (red). Giantin staining was used to delimitate the Golgi region (green). Golgi versus total cell fluorescence ratio (as a percentage) was measured for CI-MPR and TGN46. Two independent experiments (A, siCtrl, $n=317$ cells; siVAMP2/3/8, $n=254$ cells; B, siCtrl, $n=336$ cells; siVAMP2/3/8, $n=298$ cells). NS, non significant (Bonferroni's multiple comparison test). Scale bars: 10 μ m. Means $\pm$ s.e.m. are shown.

polyclonal anti-CALM (Santa Cruz Biotechnology, catalog no. sc-6433, 1:1000 for western blotting); mouse monoclonal anti- β -actin (Sigma, catalog no. A5316, 1:5000 for western blotting); mouse monoclonal anti-dynamin (BD Transduction Laboratories, catalog no. 610246, 1:1000 for western blotting); secondary antibodies conjugated to Alexa Fluor 488, Cy3, Cy5 or horseradish peroxidase (HRP) (Beckman Coulter or Invitrogen). The mouse monoclonal anti-STxB antibody 13C4 was purified from hybridoma cells (ATCC CRL-1794), and the rabbit polyclonal anti-VAMP3 antibody (used at 1:4000 for western blotting) was a gift from Thierry Galli (Institut Jacques Monod, Paris, France). 2-deoxy-D-glucose and sodium azide were purchased from Sigma.

Cell culture

HeLa cells were grown at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) high glucose Glutamax (Invitrogen) supplemented with 10% fetal calf serum (FCS), 0.01% penicillin-streptomycin, and 5 mM pyruvate. Genome-edited Rab5-GFP-expressing HeLa cells were a gift from Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), and were grown described above. HeLaM cells stably expressing N-terminally HA-tagged VAMP2, VAMP3 and VAMP8 were kindly provided by Andrew Peden (Department of Biomedical Science, University of Sheffield, UK). These were grown as described above in medium that was supplemented with 0.5 mg/ml geneticin.

Depletion of cellular ATP

Cellular ATP was depleted as previously described (Römer et al., 2007; Zha et al., 1998). Briefly, cells were incubated for 15 to 20 min at 37°C in PBS⁺⁺

supplemented with 10 mM 2-deoxy-D-glucose and 10 mM Na₃. PBS⁺⁺ was prepared by adding CaCl₂ (0.5 mM) and MgCl₂ (1 mM) in PBS.

DNA constructs and transfection

Expression plasmids for GFP-VAMP1 (Glenn Randall, Department of Microbiology, University of Chicago, USA), GFP-VAMP2 (Kazushi Kimura, Institute of Medical Life Sciences, Mie University, Japan), GFP-VAMP3, GFP-VAMP4, VAMP7-pHluorin and GFP-VAMP8 (Thierry Galli), VAMP5-GFP (Wanjin Hong, Institute of Molecular and Cell Biology, Singapore), GFP-Rab11 (Marino Zerial), GFP-syntaxin-3 (Serhan Karvar, Division of Gastrointestinal & Liver Diseases, University of Southern California, Los Angeles, USA), GFP-syntaxin-4 and GFP-syntaxin-5 (Jeffrey Pessin, Department of Medicine, Albert Einstein College of Medicine, New York, USA), syntaxin-7-GFP (Stefan Linder, Institut für medizinische Mikrobiologie, Virologie und Hygiene, Universitätsklinikum Hamburg-Eppendorf, Germany), syntaxin-13-GFP (Marc Coppolino, Molecular and Cellular Biology, University of Guelph, USA) were kindly provided by the indicated colleagues. A bicistronic vector encoding E-cadherin-SBP-mCherry and KDEL-streptavidin, used for the RUSH assay, was kindly provided by Franck Perez (Institut Curie, Paris, France).

For immunofluorescence and live-cell imaging experiments, plasmids were transfected with FuGene 6 (Promega) according to the manufacturer's instructions, or using the classical calcium phosphate procedure (Jordan et al., 1996). Cells were used for experiments at 16 to 24 h after transfection.

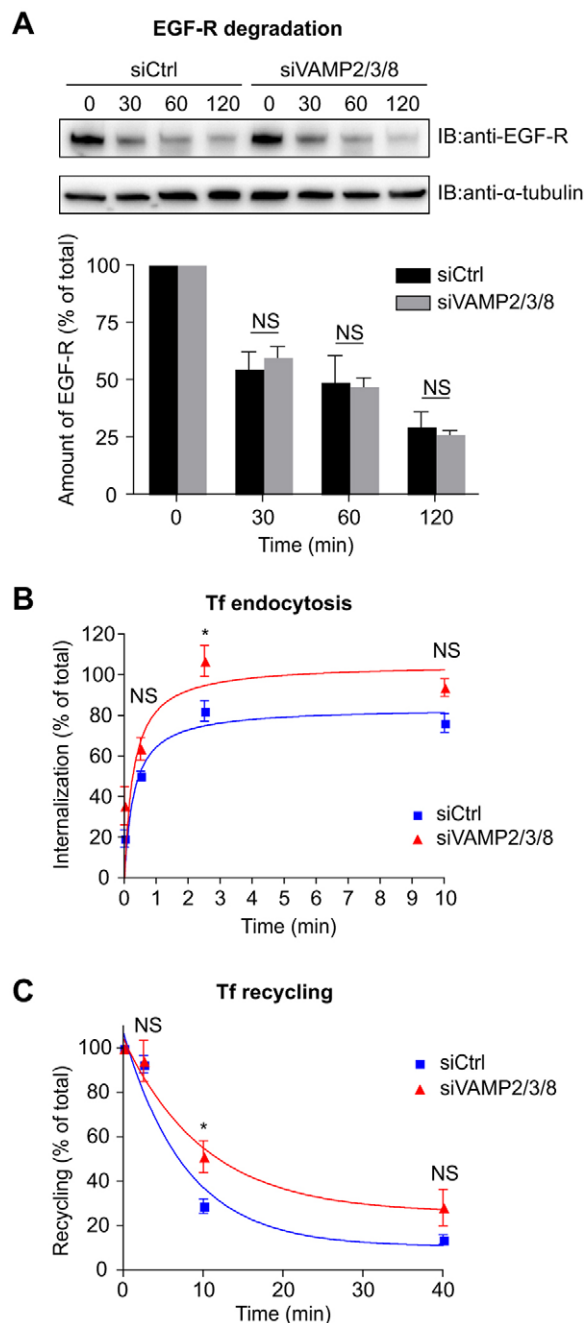


Fig. 6. EGFR and Tf trafficking in VAMP2/3/8-depleted cells. All experiments were performed on control (siCtrl) or VAMP2/3/8-depleted HeLa cells (siRNAs pool 1, siVAMP2/3/8). (A) EGFR degradation. Cycloheximide-treated cells were incubated with 100 ng/ml EGF for the indicated periods of time, and EGFR levels in cell lysates were determined by western blotting. α -tubulin was used as loading control. Quantifications show EGFR signals relative to the 0 min conditions. Three independent experiments. NS, non significant (Bonferroni's multiple comparison test). (B) Tf endocytosis. Internalization levels as a percentage of the total Tf. Six independent experiments. NS, non significant. * P <0.05 (Bonferroni's multiple comparison test). (C) Tf recycling. Levels of cell-associated Tf at different time points as a percentage of the initial Tf levels. Four independent experiments. NS, non significant. * P <0.05 (Bonferroni's multiple comparison test). Means $\pm$ s.e.m. are shown.

RNA interference

All siRNAs used in this study were purchased from Qiagen and transfected with HiPerFect (Qiagen) according to manufacturer's instructions. Experiments were performed 72 h after siRNA transfection, when protein

depletion efficiency was maximal (as shown by immunoblotting analysis with specific antibodies; routinely 80–90% depletion). For most experiments, cells were re-plated 24 h before use, according to the need of the experiment. AllStars Negative Control siRNA served as a reference point. The depletion of VAMPs was achieved with single siRNA sequences at a final concentration of 40 nM: Hs_VAMP1_7 for VAMP1 (ref. SI04347595, 5'-ACCACCCATGTGCATGAGCAA-3'); Hs_VAMP2_5 for VAMP2 (ref. SI03027241, 5'-AACAAGCGCAGCCAAGCTCAA-3'); Hs_VAMP2_4 for VAMP2 (ref. SI00103838, 5'-CCCATTAGTTCTTGT-ATCACA-3'); Hs_VAMP3_2 for VAMP3 (ref. SI00759234, 5'-CAGG-CGCTTCTCAATTTGAAA-3'); Hs_VAMP3_5 for VAMP3 (ref. SI04214924, 5'-TCGGGATTACTGTTCTGGTTA-3'); Hs_VAMP5_4 for VAMP5 (ref. SI00759304, 5'-CAGGATGCAGGCATTGCCTCA-3'); Hs_VAMP8_5 for VAMP8 (ref. SI02656773, 5'-TGGAGGGAGTTAAG-AATATTA-3'); Hs_VAMP8_4 for VAMP8 (ref. SI02656766, 5'-CTGG-TGCCTTCTCTTAAGTAA-3'). For syntaxin-16, CALM and dynamin-2 depletion, single siRNA sequences were used at a final concentration of 40 nM: for syntaxin-16, 5'-AAGCAGCGATTGGTGTGACAA-3' (Amessou et al., 2007); for CALM, 5'-AAACAGTTGGCAGACAGTTT-A-3' (Miller et al., 2011); and for dynamin-2, 5'-CTGCAGCTCATCTTC-TCAAAA-3' (ref. SI02654687).

Recombinant proteins

Recombinant wild-type STxB, STxB/Cys, and STxB-Sulf₂ were purified from bacterial periplasmic extracts as previously described (Mallard and Johannes, 2003). Periplasmic extracts were loaded on a QHP column (GE Healthcare) and eluted in a linear NaCl gradient in Tris-HCl buffer. STxB-Sulf₂-containing fractions were pooled, validated for purity by SDS-polyacrylamide gel electrophoresis, and snap frozen in liquid nitrogen before storage at -80°C . Wild-type STxB and STxB/Cys proteins were also pooled, validated for purity, and dialyzed against coupling buffer (20 mM HEPES-KOH pH 7.4, 150 mM NaCl). These proteins were subjected to coupling with the following fluorescent dyes according to the manufacturers' instructions: wild-type STxB with NHS-activated Cy3, Cy5 (GE Healthcare) or Alexa Fluor 488 (Invitrogen); STxB/Cys with maleimid-activated Cy3 (GE Healthcare) or Alexa 488 (Invitrogen). Free dye was removed by gel filtration on PD-10 columns (GE Healthcare), and the fluorescently labeled proteins were snap frozen in liquid nitrogen before storage at -80°C .

Light microscopy

For immunofluorescence studies, cells were maintained at 37°C during the full duration of the experiment and during fixation (4% paraformaldehyde for 10 min) to preserve the integrity of STxB-induced tubules. For antibody uptake assays, cells were placed on ice after different time points to stop endocytosis. In this case, fixation (4% paraformaldehyde) was performed for 5 min on ice, followed by 10 min at room temperature. After quenching with 50 mM NH_4Cl and permeabilization with saponin (0.5% saponin, 2% BSA in PBS), cells were incubated with primary and secondary antibodies, and mounted with Mowiol. For immunofluorescence with anti-HA antibody, cells were permeabilized with 0.5% saponin and 5% FCS.

Fixed samples were imaged with a Nikon A1R confocal microscope equipped with a CFI Plan Apo VC 60 $\times$ NA 1.4 oil immersion objective, when not specified otherwise. Wide-field images were acquired on a Leica DM 6000B epifluorescence inverted microscope equipped with a HCX PL Apo 63 $\times$ NA 1.40 oil immersion objective and an EMCCD camera (Photometrics CoolSNAP HQ).

For live-cell imaging, cells were grown to subconfluence on FluoroDish chambers with integrated glass coverslips (World Precision Instruments). All observations were made at 37°C and 5% CO_2 . Images were acquired on spinning disk confocal devices (Nikon) equipped with EMCCD cameras (Photometrics CoolSNAP HQ2). Montages and movies were prepared with ImageJ or Fiji (NIH) and MetaMorph Software.

STxB sulfation

The sulfation assay was performed as previously described (Amessou et al., 2006). Briefly, cells were seeded in 24-well dishes. On the day of the experiment, cells were incubated for 90 min at 37°C in DMEM without sulfate (Invitrogen), and for 30 min on ice with 1 μM STxB-Sulf₂ in DMEM without

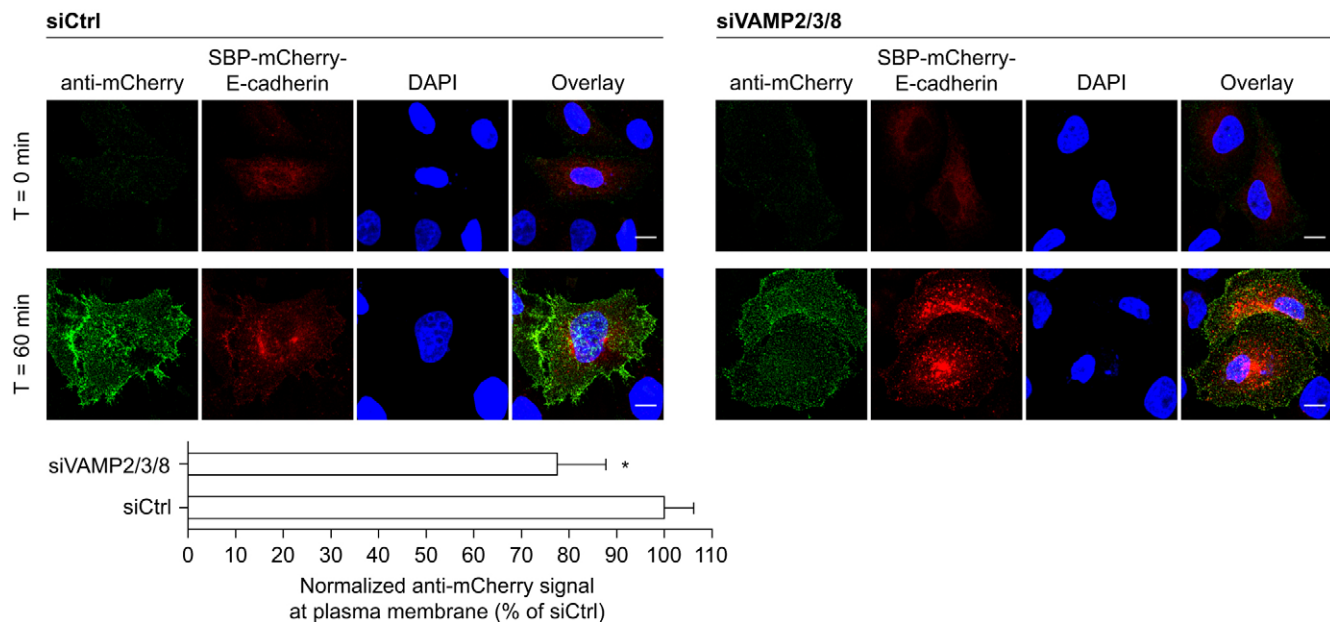


Fig. 7. Anterograde E-cadherin trafficking in VAMP2/3/8-depleted cells. Anterograde trafficking of E-cadherin was analyzed in control (siCtrl) or VAMP2/3/8-depleted HeLa cells (siRNAs pool 1, siVAMP2/3/8), using the RUSH system. After release from the ER, SBP-mCherry-E-cadherin was detected at the cell surface with anti-mCherry antibodies. After 60 min, the relative means ($\pm$ s.e.m.) of anti-mCherry fluorescence per unit area were quantified for control ($n=66$ cells) and VAMP2/3/8-depleted cells ($n=51$ cells), and normalized according to total SBP-mCherry-E-cadherin expression in each cell. Two independent experiments. * $P<0.05$ (Bonferroni's multiple comparison test). Note the significant decrease of SBP-mCherry-E-cadherin cell surface signal in VAMP2/3/8-depleted cells. Scale bars: 10 μ m.

sulfate. After washing, cells were shifted for 20 min to 37°C in DMEM containing 400 μ Ci/ml (14.8 MBq/ml) [35 S]sulfate as the sole sulfate source (Perkin-Elmer). Cells were lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.5% SDS in PBS) and STxB-Sulf₂ was immunoprecipitated for 2 h at 4°C with 13C4 antibody on Protein-G-Sepharose beads (GE Healthcare). After immunoprecipitation, unbound proteins were precipitated in 10% trichloroacetic acid (TCA) and kept at 4°C for scintillation counting. The beads were washed extensively with 50 mM Tris-HCl pH 8, after which STxB-Sulf₂ was released from the beads by boiling in sample buffer. Samples were loaded on SDS-polyacrylamide gels. After migration, gels were fixed, dried and exposed overnight to a storage phosphor screen (Molecular Dynamics). Autoradiography was revealed using Typhoon Trio device (GE Healthcare). The signal of sulfated STxB bands was quantified by ImageJ (NIH) and normalized to the total sulfation levels. Total sulfation levels were determined from TCA-precipitated proteins. After filtration through a glass-fiber filter (Whatman), radioactivity was quantified with a Wallac 1450 MicroBeta liquid scintillation counter (Perkin-Elmer).

Intoxication

The intoxication assay with STx-1 holotoxin was performed as previously described (Stechmann et al., 2010). Briefly, 24 h before the assay, 20,000 cells per well were seeded in 96-well plates (Thermo Scientific Nunc). On the day of the experiment, cells were challenged with increasing doses of STx-1 holotoxin for 1 h. Protein biosynthesis was determined for another 1 h by measuring the incorporation of radiolabeled methionine into acid-precipitable material, using a Wallac 1450 MicroBeta liquid scintillation counter (Perkin-Elmer). The mean percentage of protein biosynthesis was determined and normalized from duplicate wells. All values were expressed as means $\pm$ s.e.m. Data were fitted with Prism v4.0 software (Graphpad Inc., San Diego, CA) to obtain the 50% effective toxin concentration (EC₅₀). EC₅₀ values and protection factors (EC₅₀ of interference condition/EC₅₀ of control condition) were determined by the nonlinear regression dose-response EC₅₀ shift equation. The goodness of fit was assessed by assessing the coefficient of determination and confidence intervals.

Antibody uptake

HeLaM cells stably expressing HA-tagged VAMP3 were transfected with control siRNA or siRNA against CALM and incubated on ice for 45 min

with Alexa-Fluor-488-labeled anti-HA (1:100 dilution) in PBS⁺⁺ with 5% FCS, with or without 200 nM STxB/Cys-Cy3, and then incubated for 5 or 15 min at 37°C in PBS⁺⁺ with 5% FCS. Endocytosis was stopped on ice, and cells were washed with ice-cold PBS⁺⁺. Reduction of the remaining plasma membrane signal was achieved with four acid washes of 2 min with pH 2.0 DMEM containing 25 mM sodium acetate. Neutralization between each acid wash was performed with pH 10.0 DMEM containing 25 mM Tris. Samples were washed again with ice-cold PBS⁺⁺, and then incubated for 10 min on ice with 10 μ g/ml Alexa-Fluor-647-labeled wheat germ agglutinin (WGA-Alexa647) in order to label the plasma membrane. After three washes with ice-cold PBS⁺⁺, cells were finally fixed (4% paraformaldehyde), quenched with NH₄Cl 50 mM and mounted in Mowiol before imaging by confocal microscopy.

Flow cytometry

siRNA-transfected cells were detached using 4 mM EDTA and incubated for 30 min on ice with Tf (10 μ g/ml) and STxB/Cys (1 μ M), respectively tagged with Alexa Fluor 647 or 488, in PBS⁺⁺ containing 0.2% BSA. After washing in ice-cold PBS, fluorescence was measured with a LSR-II flow cytometer (BD Biosciences). Single-stained samples were used to verify that the fluorescence from each fluorophore was only detected in the expected channel.

EGFR degradation

HeLa cells were seeded at 600,000 cells per well in six-well plates, and after 24 h, incubated for 1 h at 37°C with 100 μ g/ml cycloheximide in serum-free DMEM, to inhibit protein biosynthesis. Subsequently, the medium was replaced by pre-warmed serum-free DMEM containing 100 μ g/ml cycloheximide, 0.2% BSA, and 100 ng/ml EGF to stimulate EGFR endocytosis and degradation. After incubation for 0, 30, 60 or 120 min at 37°C, cells were placed on ice, washed with ice-cold PBS, lysed and EGFR levels were analyzed by western blotting.

Transferrin endocytosis

The Tf endocytosis assay was performed essentially as previously described (Amessou et al., 2006). Briefly, human diferric transferrin (Tf) was biotinylated using NHS-SS-biotin (Pierce). siRNA-transfected HeLa cells were detached with 4 mM EDTA in PBS and incubated for 30 min on ice with 20 μ g/ml Tf-SS-biot in PBS⁺⁺ supplemented with 5 mM glucose and

0.2% BSA. After washing in the same buffer, the cells were aliquoted (150,000 cells per data point) and incubated at 37°C for the indicated times. Cells were placed on ice, biotin was removed from cell-surface-exposed Tf by incubation for 30 min at 4°C with 200 mM MESNA in TNB buffer (50 mM Tris-HCl pH 8.8, 100 mM NaCl, 0.2% BSA). The reaction was quenched for 30 min with 300 mM iodoacetamid in TNB buffer, cells were lysed in 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.2% BSA, 0.1% SDS, 1% Triton X-100 before being loaded onto ELISA plates (NUNC) coated with anti-Tf antibodies (mAb H68.4, 1:500 dilution). Biotinylated Tf was quantified with streptavidin-POD (Roche Applied Sciences), using o-phenylenediamine peroxidase substrate (Sigma-Aldrich). The absorbance was measured at 490 nm after stopping the reaction with 3 M H₂SO₄. Endocytosis was expressed as the percentage of internalized Tf (protected from MESNA treatment).

Transferrin recycling

Cells were incubated for 40 min at 37°C with Tf-SS-biot (60 µg/ml) in PBS⁺⁺ supplemented with 5 mM glucose and 0.2% BSA. After washing, cells were aliquoted (150,000 cells per data point), placed for the indicated times at 37°C in PBS⁺⁺ supplemented with 5 mM glucose and 0.2% BSA in the presence of a 50-fold molar excess of non-biotinylated holo-Tf (Sigma-Aldrich), transferred on ice, washed in ice-cold PBS⁺⁺, and lysed in 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.2% BSA, 0.1% SDS and 1% Triton X-100. The amount of cell-associated biotinylated Tf was determined by ELISA, as in the endocytosis assay.

Anterograde E-cadherin transport

The Retention Using Selective Hooks (RUSH) system was used to quantify anterograde transport of E-cadherin, as previously described (Boncompain et al., 2012). HeLa cells were transfected with siRNAs, and seeded after two days into 24 well plates. 4 h after plating, cells were transfected with a bicistronic vector encoding SBP-mCherry-E-cadherin and KDEL-streptavidin, using the calcium phosphate procedure (Jordan et al., 1996), and incubated for another 16 h at 37°C. At zero time point, 40 µM biotin was added into the culture medium. After the indicated times, cells were put on ice and incubated for 30 min in PBS⁺⁺ containing 0.2% BSA (Sigma-Aldrich), followed by incubation on ice with anti-mCherry antibody. Cells were then fixed, and mCherry antibody was revealed using an Alexa-Fluor-488-coupled goat anti-mouse secondary antibody. For quantification, stack images were acquired, and z-projections (sum of all slices) were created. ROI were drawn around cells and the mean fluorescence of E-cadherin-SBP-mCherry detected at the cell surface with anti-mCherry antibody was measured.

Image quantifications

All image quantifications were performed with ImageJ or Fiji (NIH) and Matlab (MathWorks).

Quantification of STxB transport to the Golgi apparatus

z-stacks were acquired on images of cells in defined experimental conditions. STxB-Cy3 fluorescence intensities were measured with ImageJ software (NIH) on z-projections, either from the entire cell, or from the Golgi region, as defined by Giantin labeling. The ratio was then calculated as an index of Golgi localization.

Quantification of anti-HA antibody uptake

This quantification was performed on single z-slices. Residual plasma membrane signal of anti-HA-Alexa Fluor-488 was subtracted on each image. To do so, a mask of plasma membrane was created from the WGA-Alexa647 images. WGA-Alexa647 labeling were first automatically thresholded using the default black-and-white threshold (with dark background). Regions of interest (ROIs) corresponding to plasma membrane were drawn using the 'create selection' tool. A two-pixel enlargement was applied to all ROIs. These ROIs were then used on the Alexa Fluor 488 images in order to clear the remaining anti-HA-Alexa-Fluor-488 plasma membrane signal. The mean fluorescence intensity of internalized anti-HA-Alexa-Fluor-488 per pixel of cell area was measured, after background correction with a rolling radius of 50 pixels. The mean fluorescence intensity after 0 min endocytosis of anti-HA-Alexa-Fluor-488 (samples kept on ice) was used to

calibrate the experiment: the samples submitted to acid washes and subtraction of residual plasma membrane signal were considered as 0%, and the samples without acid washes and without subtraction of residual plasma membrane signal were considered as 100%.

Quantification of colocalization on confocal images

In order to quantify the colocalization between STxB-Cy3 and cell compartment markers (Rab5, Rab11, TfR and LAMP1), an object-based method based on centers of mass-particles coincidence was used, as implemented in the JACoP plugin of ImageJ (Bolte and Cordelières, 2006). Before the plugin application, images were treated to remove big fluorescent patches that could interfere with the quantification (like Golgi labeling). The results were expressed as the percentage of colocalized STxB-Cy3 spots (with the green channel) over the total number of STxB-Cy3 spots in the cytoplasmic area.

Statistical analyses

All statistical analyses were performed using Prism v4.0 software (Graphpad Inc., San Diego, CA). When possible, data were tested for Gaussian distribution with Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lillie for *P*-value). In case of non-Gaussian distribution, nonparametric tests were performed: a two-tailed Mann-Whitney *U*-test if there were only two conditions to compare, or a one-way ANOVA (Kruskal-Wallis test) with a Dunn's test if there were more than two data groups to compare. In case of Gaussian distribution, parametric tests were carried on: two-tailed *t*-test for the comparison of the means if there were only two conditions to compare, parametric one-way ANOVA with a Bonferroni test if there were more than two data groups to compare. Significance of mean comparison is represented on the graphs with asterisks. All error bars correspond to the s.e.m. No statistical method was used to predetermine sample size.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.-F.R. and L.J. conceived and designed the study. H.-F.R., M.D.G.-C. and V.C. performed the experiments and collected the data. H.-F.R. performed the screening of libraries, STxB transport and sulfation assays, live-cell imaging, antibody uptake assays, immunofluorescence experiments, EGFR degradation assays, Tf endocytosis and recycling assays, RUSH experiments, flow cytometry analyses and western blotting. M.D.G.-C. performed intoxication assays. V.C. produced fluorescently labeled STxB. All authors and C.L. critically revised the manuscript. H.-F.R. and L.J. wrote the paper.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.171116/-/DC1>

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