Endophilin-A2-dependent tubular endocytosis promotes plasma membrane repair and parasite invasion

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ABSTRACT
Endocytosis of caveolae has previously been implicated in the repair of plasma membrane wounds. Here, we show that caveolin-1-deficient fibroblasts lacking caveolae upregulate a tubular endocytic pathway and have a reduced capacity to reseal after permeabilization with pore-forming toxins compared with wild-type cells. Silencing endophilin-A2 expression inhibited fission of endocytic tubules and further reduced plasma membrane repair in cells lacking caveolin-1, supporting a role for tubular endocytosis as an alternative pathway for the removal of membrane lesions. Endophilin-A2 was visualized in association with cholera toxin B-containing endosomes and was recruited to recently formed intracellular vacuoles containing Trypanosoma cruzi, a parasite that utilizes the plasma membrane wounding repair pathway to invade host cells. Endophilin-A2 deficiency inhibited T. cruzi invasion, and fibroblasts deficient in both caveolin-1 and endophilin-A2 did not survive prolonged exposure to the parasites. These findings reveal a novel crosstalk between caveolin-1 and endophilin-A2 in the regulation of clathrin-independent endocytosis and plasma membrane repair, a process that is subverted by T. cruzi parasites for cell invasion.

KEY WORDS: Endocytosis, Endophilin, Plasma membrane repair

INTRODUCTION
For injured cells to survive, plasma membrane (PM) wounds must be rapidly repaired. After several decades of investigation, it is now clear that eukaryotic cells do not reseal spontaneously. Wound repair is fast and critically dependent on the influx of extracellular Ca2+, which triggers a sequence of steps culminating in the repair of plasma membrane wounds. Here, we show that caveolin-1-deficient fibroblasts lacking caveolae upregulate a tubular endocytic pathway and have a reduced capacity to reseal after permeabilization with pore-forming toxins compared with wild-type cells. Silencing endophilin-A2 expression inhibited fission of endocytic tubules and further reduced plasma membrane repair in cells lacking caveolin-1, supporting a role for tubular endocytosis as an alternative pathway for the removal of membrane lesions. Endophilin-A2 was visualized in association with cholera toxin B-containing endosomes and was recruited to recently formed intracellular vacuoles containing Trypanosoma cruzi, a parasite that utilizes the plasma membrane wounding repair pathway to invade host cells. Endophilin-A2 deficiency inhibited T. cruzi invasion, and fibroblasts deficient in both caveolin-1 and endophilin-A2 did not survive prolonged exposure to the parasites. These findings reveal a novel crosstalk between caveolin-1 and endophilin-A2 in the regulation of clathrin-independent endocytosis and plasma membrane repair, a process that is subverted by T. cruzi parasites for cell invasion.
Helenius, 2002) is a form of clathrin-independent endocytosis that mediates the repair of different forms of PM injury (Andrews et al., 2014; Corrotte et al., 2013).

B lymphocytes, which do not form morphologically distinct caveolae (Fra et al., 1994), also reseal after injury with SLO by a process involving lysosomal exocytosis followed by endocytosis (Miller et al., 2015). Interestingly, SLO-permeabilized B cells upregulate a tubular endocytic pathway (Miller et al., 2015), raising the possibility that, when proteins necessary for the assembly of caveolae are absent, lipid raft PM microdomains may be mobilized for internalization in the form of larger tubule-shaped endosomes. In this study we have extended our investigation of PM repair in caveolae-deficient cells by examining mouse embryonic fibroblasts (MEFs) derived from Cav1 knockout (KO) mice, in parallel with MEFs from wild-type (WT) littermates. Our results revealed that in the absence of Cav1, the Bin-Amphiphysin-Rvs (BAR) domain-containing protein endophilin-A2 (EndoA2) assumes a central role in regulating a tubular endocytic pathway that promotes PM repair. Consistent with the extensive functional similarities previously identified between PM repair and T. cruzi invasion, we show that recruitment of EndoA2 to tubular PM invaginations plays a critical role in the mechanism by which the intracellular protozoan parasite T. cruzi invades host cells.

RESULTS

Cav1 knockout MEFs have reduced PM repair capacity

To further investigate the mechanism of caveolae-independent PM repair detected in B cells (Miller et al., 2015), we used MEFs derived from WT and Cav1 KO littermate mice (Razani et al., 2001) and performed 5 min SLO wounding assays, followed by staining with the membrane-impermeable dye propidium iodide (PI) and flow cytometry analysis to assess the extent of PM repair (Idone et al., 2008b). In the absence of Ca²⁺, a condition that does not allow PM repair, WT and Cav1 KO MEFs were equally susceptible to permeabilization with 50 ng/ml SLO, as indicated by the similar percentages of cells with high PI staining (88.2% for Cav1 KO and 84.7% for WT; Fig. 1A, no Ca²⁺). In the presence of Ca²⁺, a condition permissive for PM repair, the high-PI cell populations were markedly reduced (9% for Cav1 KO and 12.9% for WT), indicating that both cell types were able to remove SLO pores, blocking entry of the membrane-impermeable dye (Fig. 1A, +Ca²⁺). However, a rightward shift in the low-PI population...
Cholera toxin B is taken up predominantly through caveola-like vesicles in WT MEFs and through tubular endosomes in Cav1 KO MEFs

To investigate whether the differences observed in the resealing capacity of SLO-permeabilized WT or Cav1 KO MEFs were associated with changes in endocytic carriers, we used transmission electron microscopy (TEM) to examine recently formed intracellular compartments containing cholera toxin B (CTxB), which binds to the GM1 glycosphingolipid that is concentrated on lipid rafts and caveolae (Parton, 1994; Pelkmans and Helenius, 2002). CTxB is a well-established cargo of clathrin-independent endocytosis (Boucrot et al., 2015; Renard et al., 2015), but can also enter cells through additional endocytic pathways (Stoeber et al., 2012). Cells were treated with or without SLO for 1 min in the presence of CTxB-gold conjugates, and after fixation and TEM processing, membrane-bound compartments containing the tracer were quantified on randomly-acquired images (Fig. 2A). Four categories of endocytic compartments containing CTxB were identified: caveola-like vesicles smaller than 100 nm (<100 nm), vesicles larger than 100 nm (>100 nm), tubular endosomes, and clathrin-coated vesicles (CCV). Consistent with previous reports (Parton, 1994), in WT MEFs, CTxB was most frequently detected within uncoated caveola-like vesicles smaller than 100 nm. Some CTxB was found in uncoated tubular endosomes, whereas markedly lower amounts were present in larger vesicles and in CCVs (Fig. 2A,B, WT). Thus, although CTxB can enter cells through various endocytic routes (Ewers and Helenius, 2011; Torgersen et al., 2001), in the MEFs used in this study, caveola-like vesicles smaller than 100 nm are the preferred pathway for endocytosis after a short 1 min incubation period.

Strikingly, a marked change was observed in the morphology of CTxB endocytic carriers in Cav1 KO MEFs. In these cells that lack caveolae, most of the tracer was present inside elongated tubular compartments, often visualized in continuity with the PM (Fig. 2A, Cav1 KO, arrowheads). Consistent with the lack of morphologically distinct caveolae, only very few vesicles smaller than 100 nm containing CTxB were found in Cav1 KO MEFs, with or without SLO treatment. This decrease in the number of caveola-like vesicles in Cav1 KO MEFs was accompanied by a dramatic increase in tubular endocytic compartments containing CTxB, whereas only a very small fraction of the CtxB was detected in CCVs (Fig. 2A,B, Cav1 KO). These results suggest that Cav1 deficiency in MEFs is associated with upregulation of a population of clathrin-independent tubular endosomes, perhaps as a compensatory mechanism for the absence of caveolae, as previously observed in B lymphocytes (Miller et al., 2015).

EndoA2 is required for the scission of tubular endosomes containing CTxB and for PM repair in Cav1 KO MEFs

Next, we investigated the role in PM repair of the tubular endocytic pathway upregulated in the absence of caveolae. To this end, we used siRNA in both WT and Cav1 KO MEFs to inhibit the expression of proteins previously reported to participate in tubular endocytosis, such as galectin-3 (Lakshminarayan et al., 2014) and endophilins (Ambroso et al., 2014; Renard et al., 2015). After confirming that siRNA treatment decreased expression of these proteins (Fig. 3A; Fig. S1B,D,F), we determined the ability of WT and Cav1 KO MEFs to reseal their PM after permeabilization with SLO for 2 min. RNAi-mediated knockdown of galectin-3, endophilin-A1 and endophilin-A3 had no effect on the typical PI-staining pattern of each cell type in the flow cytometry assay, reflecting their PM repair capacity (Fig. S1A,C,E). We also tested the involvement of clathrin-dependent endocytosis by reducing levels of clathrin heavy chain expression, which inhibited endocytosis of biotinylated surface proteins but had no impact on PM repair (data not shown).

On the other hand, RNAi-mediated knockdown of EndoA2 (Fig. 3A) significantly increased the number of PI-positive Cav1 KO cells when compared to controls, accentuating the PM repair defect observed in these cells. In contrast, no consistent inhibition of PM repair was observed in WT MEFs after reducing EndoA2 expression levels (Fig. 3B). This result suggested that EndoA2 might be required for PM repair only in Cav1 KO MEFs, possibly as a consequence of the observed shift from caveolar to tubular endocytosis (Fig. 2B). To investigate this possibility, we used TEM to examine CTxB-carrying tubular endocytic structures generated within 1 min in WT and Cav1 KO MEFs that had been previously treated or not with EndoA2 siRNA. In agreement with the results discussed above (Fig. 2B), significantly more CTxB-containing tubular endosomes were observed in Cav1 KO MEFs when compared to WT MEFs. Notably, treatment with EndoA2 siRNA significantly increased the number of CTxB-containing tubular endosomes visualized in Cav1 KO MEFs, but not in WT MEFs (Fig. 3D, total tubules). Importantly, EndoA2-deficient Cav1 KO MEFs contained a significantly larger number of tubular endosomes that were ‘open’, in direct continuity with the PM, when compared to those in WT MEFs and Cav1 KO cells treated with control siRNA (Fig. 3C, arrows; Fig. 3D, open tubules). In both WT and Cav1 KO MEFs, the numbers of CTxB-containing tubular endosomes not visualized in direct continuity with the PM (Fig. 3D, apparently closed tubules) were similar in cells treated or not with EndoA2 siRNA, suggesting that open tubules accounted for the overall increase in the total number of CTxB-carrying tubular endosomes observed in EndoA2-depleted Cav1 KO MEFs (Fig. 3D). Thus, inhibition of EndoA2 expression appeared to either increase the formation of tubular endosomes or to inhibit their fission from the PM, resulting in the accumulation of open tubular endocytic compartments at the PM.
To distinguish between these two possible scenarios, we incubated WT and Cav1 KO MEFs with Alexa Fluor 488–CTxB for 10 min, followed by quenching of the extracellular fluorescence and detection of the internalized toxin by flow cytometry. The levels of CTxB endocytosis in WT MEFs were similar in cells treated with either control or EndoA2 siRNA, with or without SLO wounding (Fig. 3E, WT). This result indicates that EndoA2 expression does not influence CTxB endocytosis in WT MEFs, in agreement with the relatively low numbers of CTxB-positive tubular endosomes observed in this cell type (Fig. 2B). However, in Cav1 KO MEFs, EndoA2 siRNA treatment inhibited CTxB endocytosis when compared to that in cells treated with control siRNA (Fig. 3E, Cav1 KO). This decrease was accentuated in a dose-dependent manner by exposure to SLO, suggesting that an EndoA2-dependent form of endocytosis is specifically mobilized when Cav1 KO MEFs are injured with SLO. Taken together with the inhibition of PM repair caused by EndoA2 depletion specifically in Cav1 KO MEFs (Fig. 3B), these results suggest that Cav1 KO cells upregulate a tubular endocytic pathway that requires EndoA2 for fission from the PM, a function necessary for the removal of lesions and cell resealing.

To examine the interaction of EndoA2 with cargo internalized during the brief periods of time involved in PM repair (Idone et al., 2008b; Steinhardt et al., 1994), we performed immunofluorescence
assays using antibodies against EndoA2 in cells incubated with fluorescent CTxB for 15 s. In both WT and Cav1 KO MEFs, endogenous EndoA2 was detected in a punctate pattern on the ventral surface of cells, as previously reported (Boucrot et al., 2015). In WT MEFs, a few EndoA2 and CTxB puncta appeared juxtaposed, in a ‘strawberry’ pattern where CTxB puncta were capped by EndoA2 (Fig. 4A, arrowheads). Cav1 KO MEFs, however, had significantly higher levels of these partially colocalized CTxB–EndoA2 structures (Fig. 4A,B). In light of the data discussed above and previous reports (Boucrot et al., 2015), these immunofluorescence results suggest that the juxtaposed CTxB–EndoA2 structures that were more frequently detected in Cav1 KO MEFs may correspond to EndoA2-dependent tubular endosomes.
EndoA2 promotes cell invasion by *Trypanosoma cruzi* and is recruited to nascent parasite-containing intracellular vacuoles

Previous studies have shown that the intracellular protozoan *T. cruzi* wounds the PM of host cells, triggering a repair process that is subverted by the parasites for invasion (Fernandes et al., 2011). Given that several interventions that inhibit PM repair also inhibit *T. cruzi* invasion (Chakrabarti et al., 2003; Fernandes et al., 2011; Fernandes et al., 2007; Reddy et al., 2001), we examined the impact of EndoA2 depletion on the susceptibility of WT and Cav1 KO MEFs to infection by these parasites. We first exposed both cell types to infective stages of *T. cruzi* for increasing periods of time and found that Cav1 KO MEFs were slightly less susceptible than WT MEFs to infection (Fig. 5A). Both cell types were able to repair *T. cruzi*-induced PM damage, because no host cell loss was observed over an assay period of 3 h (Fig. 5B). However, when siRNA was used to silence EndoA2 expression, a significant reduction in invasion was observed in both WT and Cav1 KO MEFs after 1 h of exposure to the parasites (Fig. 5C, 1 h). During this period, there was also no significant cell loss in MEF cultures treated with control or EndoA2 siRNA (Fig. 5D, 1 h). After a 2 h infection period, a marked reduction in parasite invasion was again observed in WT MEFs treated with EndoA2 siRNA, and host cells were not lost from cultures treated with control or EndoA2 siRNA (Fig. 5D, WT 2 h). In contrast, treatment with EndoA2 siRNA followed by 2 h of exposure to *T. cruzi* resulted in marked host cell loss from Cav1 KO cultures, preventing the quantification of parasite invasion at this time point (Fig. 5D, Cav1 KO 2 h). Thus, whereas MEFs lacking Cav1 are partially less susceptible than WT MEFs to *T. cruzi* infection, deficiency in both Cav1 and EndoA2 strongly impairs the cells’ ability to survive during the parasite infection process. Considering that Cav1 KO cells require EndoA2 expression to successfully repair most SLO lesions, as discussed above (Fig. 3B), these *T. cruzi* infection results provide independent evidence that cells lacking Cav1 become critically dependent on EndoA2 to survive PM injury – in this case inflicted by the parasites – as previously described (Fernandes et al., 2011).

The large size of *T. cruzi* trypomastigotes provided us with a good opportunity to examine EndoA2 localization during formation of the elongated, tight parasitophorous vacuoles that surround the parasites as they enter host cells. In order to detect early stages of the parasite-induced PM invaginations, we performed immunofluorescence assays of EndoA2 in WT and Cav1 KO MEFs incubated with *T. cruzi* for 15 min. Polyclonal anti-*T. cruzi* antibodies were used to
stain extracellularly-exposed portions of the parasites prior to permeabilization of the cells and EndoA2 staining, allowing us to determine the stage of parasite invasion while assessing the recruitment of EndoA2.

In both WT and Cav1 KO MEFs, parasites that were fully stained with anti-\textit{T. cruzi} antibodies and therefore considered extracellular showed no EndoA2 staining (Fig. 6, top panels). However, when partially internalized parasites were detected (Fig. 6, middle panels, arrowheads pointing to partial anti-\textit{T. cruzi} extracellular staining), the internalized portion of the parasites was clearly surrounded by EndoA2 staining in both cell types (Fig. 6, middle panels, arrows pointing to EndoA2 staining). Interestingly, faint EndoA2 staining was visible on some parasite regions that were positive for anti-\textit{T. cruzi} antibodies, suggesting that EndoA2 recruitment to the inner leaflet of the PM may be initiated prior to invasion. Fully internalized portions of the same parasites showed brighter staining with anti-EndoA2 antibodies, consistent with an accumulation of this cytosolic protein on the membrane of nascent parasitophorous vacuoles.

In several instances, fully internalized parasites showing no anti-\textit{T. cruzi} staining were detected, and in this case continuous EndoA2 staining was observed along the tight intracellular compartments that are generated during cell invasion. However, a few fully internalized parasites negative for anti-\textit{T. cruzi} staining did not react with EndoA2 antibodies (Fig. 6 WT and Cav1 KO MEFs, lower panels). This finding suggested that EndoA2 might only be transiently recruited to the \textit{T. cruzi} parasitophorous vacuole, although this is difficult to assess for certain because the \textit{T. cruzi} invasion process cannot be synchronized. These immunofluorescence results are consistent with a progressive recruitment of EndoA2 to the nascent \textit{T. cruzi}-containing parasitophorous vacuoles, followed by EndoA2 loss after the host cell invasion process is completed. Thus, combined with the requirement for EndoA2 expression for cells to become susceptible to invasion by \textit{T. cruzi}, our results identify EndoA2 as a component of the cellular machinery that is subverted by these parasites to invade host cells.

**DISCUSSION**

Previous studies from our group have revealed a role for clathrin-independent endocytosis in the mechanism by which mammalian cells repair PM wounds. In NRK cells and muscle fibers, caveolar endocytosis is stimulated when cells are mechanically injured or permeabilized with the pore-forming toxin SLO, and Cav1 depletion inhibits caveolea internalization and PM repair. Direct visualization by electron microscopy of SLO within Cav1-containing intracellular vesicles suggested that caveolar endocytosis is directly involved in the removal and subsequent intracellular degradation of PM lesions.
However, additional evidence implicating caveolae or other forms of clathrin-independent endocytosis in PM repair was needed, particularly in view of evidence that the ESCRT pathway can also promote cell resealing by removing small lesions from the PM (Jimenez et al., 2014). B lymphocytes that lack Cav1 and caveolae can still reseal their PM after SLO permeabilization, concomitantly with upregulation of a tubular endocytic pathway (Miller et al., 2015). In this study we confirmed and expanded on those previous findings, providing mechanistic evidence for a role of tubular endocytosis in PM repair in cells lacking caveolae. Importantly, we found that expression of the endocytosis regulatory protein EndoA2 (Renard et al., 2015) was required for CTxB internalization and for the resealing of SLO-permeabilized MEFs, but only in Cav1-deficient fibroblasts lacking caveolae. In these cells, the most abundant CTxB carriers detected were tubular endosomes, and their scission from the PM required EndoA2, an effect that was not associated with increased EndoA2 expression levels (Fig. 3A). Notably, independent new evidence has uncovered a striking connection between B cell function and EndoA2-mediated PM repair. An unbiased CRISPR screen identified EndoA2 as an important regulator of B cell-mediated humoral responses (Malinova et al., 2020, preprint) and B cell PM wounding and repair emerged as an antigen-triggered process that facilitates antigen uptake and presentation to T cells (Maeda et al., 2020, preprint).

Overall, our findings are in line with the reported upregulation of clathrin-independent carriers known as CLIC/GEEC in Cav1 KO MEFs (Chaudhary et al., 2014), and suggest that when Cav1 is not present to shape the PM into structurally defined caveolae, formation of tubular endosomes capable of mobilizing similar lipid raft (cholesterol and sphingolipid-enriched) PM domains may be facilitated, with important functional consequences. Interestingly, our quantitative flow cytometry-based PM repair assay suggests that EndoA2-dependent tubular endocytosis is not as efficient as caveolar internalization in removing SLO pores from the PM, as indicated by the partial permeability to PI of Cav1 KO cell populations that recovered from SLO injury (Fig. 1).

In addition to strengthening the evidence for a role of endocytosis in PM repair, our findings uncovered a crosstalk between caveolar and EndoA2-dependent tubular endocytosis, consistent with previous suggestions (Chaudhary et al., 2014; Thottacherry et al., 2019). Our results also reinforce previous evidence that the
intracellular parasite *T. cruzi* subverts the PM repair process to invade host cells (Fernandes et al., 2011). We found that Cav1 KO MEFs were slightly less susceptible to *T. cruzi* invasion, but when EndoA2 expression was silenced, parasite entry was markedly reduced in both WT and Cav1 KO MEFs. MEFs deficient in both Cav1 and EndoA2 could not survive longer periods of exposure to the parasites, in agreement with the strong PM repair defect observed in SLO-permeabilized cells under those conditions.

The PM repair and *T. cruzi* invasion processes have many properties in common. Both are initiated by Ca\(^{2+}\)-triggered lysosomal exocytosis (Reddy et al., 2001; Tardieux et al., 1994; Tardieux et al., 1992), require PM cholesterol (Fernandes et al., 2007; Idone et al., 2008a; Tam et al., 2010) but not actin polymerization (Idone et al., 2008a; Tardieux et al., 1992), and are stimulated when cells are injured or treated with extracellular sphingomyelinase (Fernandes et al., 2011; Idone et al., 2008b). These properties, including stimulation by sphingomyelinase, are also associated with MEND, a massive endocytic process detected by electrophysiological approaches (Hilgemann et al., 2018). Interestingly, a recent study (Hubert et al., 2020) confirmed earlier findings that sphingomyelinase treatment stimulates caveolar endocytosis (Corrotte et al., 2013) and suggested that enrichment in glycosphingolipids and cholesterol resulting from sphingomyelin hydrolysis may promote caveole fission from the PM through lipid phase separation mechanisms.

In order to remove PM lesions within the 12–15 s timeframe of PM repair (Idone et al., 2008b; Steinhardt et al., 1994), any endocytic uptake involved in this process would have to be fast. Notably, a fast form of tubular endocytosis (FEME) was previously identified and shown to be regulated by EndoA2 (Boucrot et al., 2015). The FEME pathway has several elements in common with the clathrin-independent tubular endocytic pathway that our study has linked to PM repair in Cav1 KO MEFs, and future studies should clarify its relationship with the CLIC/GEEC pathway (Howes et al., 2010a) found to be upregulated in Cav1 KO cells (Chaudhary et al., 2014). Proteins of the endophilin family have been shown to regulate the clathrin-independent uptake of various cargoes, including the IL2 receptor, β-adrenergic receptors and bacterial toxins (Boucrot et al., 2015; Renard et al., 2015; Chan Wah Hak et al., 2018).

Endophilins contain BAR domains capable of inducing membrane curvature, SH3 domains that bind cargo and multiple amphipathic helices proposed to support membrane scission (Boucrot et al., 2015; Gallop et al., 2006; Simunovic et al., 2017). A role for EndoA2 in fission of the tubular endosomes upregulated in Cav1 KO MEFs is supported by our quantitative ultrastructural analysis showing accumulation of CTxB-containing tubules connected to the PM and reduced CTxB internalization after EndoA2 depletion (Fig. 3). The frequent juxtaposition of CTxB and EndoA2 puncta that we observed by light microscopy in Cav1 KO MEFs (Fig. 4A) also agrees with a role for EndoA2 in endosome fission from the PM, consistent with previous studies proposing that the N-terminal α-helix of BAR proteins can induce strong membrane curvature, directly promoting membrane constriction and possibly fission (Boucrot et al., 2012; Farsad et al., 2001). Endophilins and other BAR family proteins bind dynamin through their SH3 domains, and this interaction was proposed to play a role in recruiting dynamin to endocytic vesicle necks to facilitate fission (Ferguson and De Camilli, 2012). However, a recent study showed that excess EndoA2 can intercalate between turns of the dynamin helix assembled on endosome necks, inhibiting membrane fission (Hohendahl et al., 2017). It is noteworthy, however, that previous studies found the removal of SLO toxin pores from the PM through caveolar endocytosis to be a dynamin-independent process (Corrotte et al., 2013; Idone et al., 2008b). Those findings are consistent with previous evidence indicating that clathrin-independent endocytosis is less reliant on dynamin-mediated fission (Howes et al., 2010b; Renard et al., 2015). Thus, additional studies are needed to clarify whether, in certain contexts, EndoA2 can act independently of dynamin to promote membrane fission.

In contrast to our evidence supporting a role of EndoA2 in the fission of tubular endosomes in Cav1-deficient cells, EndoA2 appeared to be gradually recruited to nascent parasitophorous vacuoles surrounding *T. cruzi* trypomastigotes during host cell entry. It is conceivable that in this scenario EndoA2 plays a predominant role in the extensive membrane deformation required for generating the large compartments surrounding these parasites during invasion. It is tempting to speculate that the ability of EndoA2 to polymerize into rigid scaffolds and block lipid diffusion, when under tension in membrane tubules (Simunovic et al., 2016), may participate in generation of the unusually tight and elongated parasitophorous vacuoles that surround intracellular *T. cruzi* parasites. Mounting evidence suggests that endophilins have broad functional roles in cells, acting as hubs for protein–protein interactions that coordinate several aspects of membrane remodeling (Kjaerulf et al., 2011; Saheki and De Camilli, 2012). The relatively slow and asynchronous *T. cruzi* host cell invasion process does not allow a precise determination of the invasion stage of individual parasites, but the fact that EndoA2 was not detected in some fully internalized parasites (Fig. 6) suggests that EndoA2 recruitment is transient, perhaps restricted to the early stages of invasion involving membrane deformation.

To our knowledge, this is the first time that a BAR domain-containing protein has been implicated in the extensive PM deformation that occurs during host cell invasion by *T. cruzi*. Our results identify EndoA2 as a novel molecular player in the PM remodeling process triggered by these parasites to enter host cells and in the compensatory tubular endocytic pathway that promotes PM repair in the absence of caveolae. In future studies it will be of great interest to investigate whether changes in lipid composition, such as those recently shown to stimulate caveolar endocytosis (Hubert et al., 2020), occur in caveolae and in tubular endosomes associated with PM repair and *T. cruzi* invasion.
Biotechnology) at 1:50 dilution; propidium iodide (PI) (P4170; Sigma-Aldrich, St. Louis, MO, USA); Alexa Fluor 488– cholera toxin B (C34775; Invitrogen); DAPI (D9542; Sigma-Aldrich) and Alexa Fluor-conjugated secondary antibodies (Invitrogen) at 1 µg/ml dilution.

**Flow cytometry PM repair assay**

Histidine-tagged streptolysin-O (SLO) carrying a cysteine deletion that eliminates the need for thiol activation (provided by R. Tweten, University of Oklahoma, Norman, OK, USA), was expressed and purified as described in Idone et al. (2008b) and stored in 1 mg/ml aliquots at ~80°C. For wounding with SLO, WT or Cav1 KO MEFs (treated or not with siRNA for 24–72 h) were trypsinized (0.25% trypsin at 37°C for ~1–5 min) and split in two different suspensions in DMEM (with or without Ca2+) at the concentration of 1×10^6 cells/ml. Aliquots of each cell suspension (0.1–0.2 ml) were incubated on ice with various concentrations of SLO for 5 min, transferred to a 37°C water bath for various time points (~1 min) to induce pore formation followed by PM repair, and then transferred back to ice. To assess the degree of PM repair, the membrane impermeant dye PI (50 µg/ml) was added, and after 5 min at least 10,000 cells were analyzed by flow cytometry (FACS Canto II, Beckton Dickinson Biosciences, Sparks Glencoe, MD, USA).

**Imaging PM repair assay**

WT and Cav1 KO MEFs (2.5×10^5) were plated on 3.5 cm glass-bottom dishes (MatTek, Ashland, MA, USA) and 24 h later were treated with 100–400 ng/ml SLO for 5 min at 4°C followed by 5 min incubation in DMEM at 37°C, washed with phosphate-buffered saline (PBS) and stained for 5 min with 50 µg/ml PI, followed by washes with PBS and fixation in 4% paraformaldehyde (PFA) for 10 min. After staining with 10 µg/ml DAPI for 10 min, cells were washed and imaged immediately in a Zeiss LSM 980 Airyscan 2 laser scanning confocal microscope using a 20× 1.0 N.A. objective (Carl Zeiss microscopy, LLC, White Plains, NY, USA). Images were acquired randomly, and more than 200 DAPI-stained cells were visually scored as PI positive or not in each condition, in triplicate experiments.

**Transcriptional silencing**

WT and Cav1 KO MEFs grown at ~50% confluence were transfected with Mission siRNA transfection reagent (Sigma-Aldrich) and control, endophilin-A1, endophilin-A2, endophilin-A3, galectin-3 or clathrin heavy chain Silencer Select siRNA (Ambion, Life Technologies, Austin, TX, USA) and 24 h later were treated with 100 ng/ml Alexa Fluor 488–CTxB at 4°C for 20 min, and then for 5 min at 4°C for with various concentrations of SLO. The cells were transfected (or not, for negative controls) to 37°C for 10 min, followed by transfer to 4°C and fixation in 4% PFA for 10 min. Flow cytometry was used to assess Alexa Fluor 488–CTxB fluorescence before and after quenching extracellular fluorescence by adding 10 µg/ml rabbit anti-Alexa Fluor 488 antibody (Life Technologies) and 1 mg/ml Trypan Blue (Sigma-Aldrich) at room temperature for 20 min. Data were analyzed using FlowJo software (Three Star Inc., Ashland, VA, USA).

**Trypanosoma cruzi infection**

Trypanosomastigotes from the *T. cruzi* Y strain were obtained from the supernatant of infected monolayers of LLC-MK2 cells, as previously described (Tardieux et al., 1992). 1×10^5 cells (WT or Cav1 KO MEFs) were plated on 3.5 cm dishes containing glass coverslips 48 h prior to experiments, and then treated or not with control or EndoA2 siRNA for 40 h prior to experiments. Coverslips with attached cells were incubated with trypanosomastigotes resuspended in 2 ml DME containing 2% FBS (multiplicity of infection 250–500) for the indicated periods of time at 37°C and washed five times with PBS to remove extracellular parasites. The coverslips were then fixed in 4% PFA for 15 min, washed three times with PBS and incubated for 15 min with 50 mM NH4Cl. Parasite and host cell DNA were stained for 10 min with 10 µM DAPI, and extracellular parasites were stained by incubation with rabbit anti-*T. cruzi* polyclonal antiserum (1:1000) for 30 min followed by anti-rabbit Alexa Fluor-conjugated secondary antibodies (Invitrogen). The number of intracellular parasites was determined by counting at least 200 cells per coverslip in triplicates, using a Nikon Eclipse E200 fluorescence microscope with a 100×1.3 N.A. oil immersion objective.

**Immunofluorescence and colocalization analysis**

**CTxB and EndoA2 localization**

WT and Cav1 KO MEFs were cultured to 50% confluency on coverslips, pre-incubated with 5 µg/ml Alexa Fluor 488–CTxB for 20 min at 4°C and treated or not with 50 ng/ml of SLO for 5 min at 4°C. Cells were then incubated at 37°C in DMEM for 15 min before fixation in 4% PFA. Cells were then quenched with 50 mM NH4Cl for 15 min, blocked with 5% goat serum for 30 min and incubated with 10 µg/ml rabbit anti-Alexa Fluor 488 antibodies (Life Technologies) for 1 h to quench extracellular Alexa Fluor 488–CTxB fluorescence. Cells were then permeabilized with 0.2% saponin in PBS, blocked again with 5% goat serum in PBS containing 0.2% saponin, and incubated with mouse anti-endophilin-A2 (1:50) in PBS containing 0.2% saponin and 1% BSA overnight at 4°C, followed by an anti-mouse secondary antibody conjugated to Alexa Fluor 647-labeled EndoA2 and Alexa Fluor 488–CTxB that could be visualized on all three axes were visually scored in at least 20 cells, in three independent experiments.

**Trypanosoma cruzi and EndoA2 localization**

WT and Cav1 KO MEFs were infected with *T. cruzi* trypanomastigotes for 10–15 min, fixed with 4% PFA, quenched with 50 mM NH4Cl and blocked in PBS containing 5% goat serum, followed by incubation for 45 min with rabbit anti-*T. cruzi* serum (1:1000 in PBS containing 1% BSA) and Alexa Fluor 647-conjugated anti-rabbit IgG secondary antibodies for 45 min. Cells were then permeabilized with 0.2% saponin in PBS, and blocked again with PBS containing 5% goat serum and 0.2% saponin. Cells were then incubated with mouse anti-endophilin-A2 antibodies (1:50) in PBS containing 0.2% saponin and 1% BSA overnight at 4°C, followed by 1 h incubation with Alexa Fluor 488–conjugated anti-mouse secondary antibodies and DNA staining with 10 µM DAPI. Z stack images (0.1 µm Z steps) were acquired using a confocal Leica SPX5 microscope with a 63×1.4 N.A. oil objective.

**Statistical analysis**

Results obtained from two independent groups of cells (data acquisition and sample size details as provided above) were compared using unpaired,
two-tailed Student’s t-tests (Prism, GraphPad software). P-values <0.05 were considered significant. No samples were excluded from the analysis.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M. Corrotte, N.W.A.; Methodology: M. Corrotte, N.W.A.; Formal analysis: M. Corrotte, F.Y.M., N.W.A.; Investigation: M. Corrotte, M. Cerasoli, F.Y.M., N.W.A.; Writing - original draft: M. Corrotte; Writing - review & editing: M. Corrotte, M. Cerasoli, F.Y.M., N.W.A.; Supervision: N.W.A.; Project administration: N.W.A.; Funding acquisition: N.W.A.

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