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# Caveolae govern plasma membrane mechanics to protect cells against EDIN B-induced transcellular tunnel formation and lethality from *S. aureus* septicaemia

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## Abstract

The spontaneous opening of large transendothelial cell macroaperture (TEM) tunnels can accompany leukocyte diapedesis and is triggered by bacterial exoenzymes that inhibit RhoA-driven cytoskeleton contractility. Modelling the dynamics of TEM via a theoretical framework used for soft matter physics allowed us to depict the essential driving forces at play on the membrane to enlarge TEMs. In this study, we conducted multidisciplinary experiments to characterize the role respectively played by cavin-1-structured caveolae and non-caveolar caveolin-1 in plasma membrane mechanics and identify their functional effects on TEM size. The results pointed towards

50 a contributing role for non-caveolar caveolin-1 in the membrane bending rigidity, a mechanical  
51 parameter we quantified in a model system of tubes pulled from plasma membrane spheres.  
52 Depletion of cavin-1-structured caveolae showed no effect on membrane rigidity, whereas caveolae  
53 controlled cell height favouring TEM nucleation. Hence, caveolae confer protection against  
54 exoenzyme EDIN-B in mice with staphylococcal septicaemia.

## 57 MAIN TEXT

### 60 Introduction

62 The endothelium lining the inner surface of the vasculature is an active monolayer that senses  
63 haemodynamic forces, enabling proper control of plasma membrane mechanical properties and  
64 barrier function (1, 2). Regulation of the endothelial barrier involves paracellular exchanges at both  
65 cell–cell junctions (3) and less-characterized transcellular exchanges realized via membrane pores  
66 that are 60-80 nm in diameter and large gaps between transendothelial cells that are several  
67 micrometres wide (4). Transcellular pores, which contribute to aqueous humour outflow and the  
68 diapedesis of leukocytes, show homology with transendothelial cell macroaperture (TEM) tunnels,  
69 the formation of which is triggered by several bacterial exoenzymes and toxins that breach the  
70 endothelial barrier, inducing oedema and haemorrhage, as well as promoting systemic  
71 dissemination of *Staphylococcus aureus* (5–7).

73 Large-scale membrane deformation induced by pathogenic bacteria and their toxins is key to the  
74 aetiology of infections. A group of exotoxins from pathogenic bacteria can trigger TEM tunnels  
75 with typical diameters ranging from 10 to 20  $\mu\text{m}$  (8–10). This group of TEM tunnel-forming toxins  
76 includes *Clostridium botulinum* exoenzyme C3 (ExoC3), which is representative of bacterial ADP  
77 ribosyltransferases that inhibit the small GTPase RhoA. ExoC3 is widely used in cell biology and  
78 is homologous to the exoenzymes EDIN (epidermal differentiation inhibitors) A, B, and C from  
79 *Staphylococcus aureus* (11) (12, 13). These exoenzymes enter host cells via nonspecific endocytic  
80 processes and translocate from vesicles into the cytosol. The TEM tunnels form spontaneously upon  
81 disruption of actin cytoskeleton-driven contractility controlled by RhoA (8, 10). Compressive  
82 forces applied to the dorsal region of cells are sufficient to bring apposing membranes into close  
83 contact, leading to the opening of TEMs in the presence or absence of EDIN (14). The magnitude  
84 of the force required to mechanically open TEMs greatly decreases when RhoA activity is inhibited  
85 (14). Therefore, inhibition of RhoA-driven cytoskeletal tension and depletion of the F-actin  
86 meshwork trigger cell spreading and a reduction in cell height, which favour the spontaneous  
87 formation of TEM tunnels (8, 15). Although TEM tunnels have not yet been visualized in vivo,  
88 they form *ex vivo* in the endothelium lining of the inner surface of aortas either infected with *S.*  
89 *aureus*-producing EDIN or treated with purified recombinant EDIN (8). The intravenous injection  
90 of EDIN or ExoC3 triggers vascular leakage (8, 16). Ultimately, cumulative evidence shows that  
91 EDIN favours the dissemination of *S. aureus* in host tissues (17, 18). Whether genetic factors confer  
92 protection to the host against the cytotoxic effects of EDIN-B in septicaemia remains to be  
93 determined.

95 Applying concepts discovered via soft matter physics to cellular microbiology enables us to define  
96 mechanical constraints and discriminate purely physical processes from those induced by activated  
97 cellular factors (19, 20). The phenomenological analogy of TEM tunnel formation and dry patches  
98 formed on solid surfaces by viscous liquid dewetting highlights the importance of membrane  
99 tension increases in response to cell spreading, which is a critical parameter of TEM tunnel

dynamics (10, 21). Membrane tension participates in TEM nucleation and it controls the initial speed of TEM opening, and tends to be reduced as the TEM is enlarged (10, 21). Despite the theoretical prediction of a key role of membrane tension in TEM opening, the cellular mechano-regulators controlling the TEM opening phase have not yet been identified.

Caveolae are mechano-regulators of the membrane that spontaneously flatten to accommodate acute mechanical loads and thereby prevent membrane rupturing (22, 23). Caveolin complexes, which are essential components in caveolae, insert into the inner leaflet of the lipid bilayer plasma membrane (23, 24). Caveolin-1 homo-oligomers form flat discoid complexes anchored to interfaces (24). Membrane bending during caveolar invagination, which form areas 50-100 nm in diameter, requires the cavin-1 structural protein (also referred to as PTRF), which plays an established role with caveolin-1 (25–29). Membrane invaginations at caveolae are further stabilized by the assembly of a ring of dynamin-like EHD2 oligomers around the caveolar neck (30–32). In endothelial cells, the cross-talk between caveolin-1 and RhoA during actomyosin contractility leads to critical mechano-sensing of and adaptation to haemodynamic forces (33, 34). Caveolin-1-deficient mice showed an increase in the eNOS signalling critical for RhoA activation via nitration of p190RhoGAP-A, which inactivated its GTPase stimulating activity on RhoA (35–37). Cell biology and epidemiological studies have recently discovered the importance of caveolin-1 in host–pathogen interactions (38–40). Considering their critical functions in ensuring membrane mechano-relaxation in response to acute mechanical loads (22), caveolae are interesting candidates for the mechano-regulation of TEM tunnels that form upon RhoA inhibition. Together, these findings point to the importance of further characterizing caveolae and the caveolae and caveolin-1 interplay during infection and gaining more information on membrane mechano-regulation.

Here, we report the susceptibility of caveolin-1-deficient mice with septicaemia to the lethal effect of EDIN-B. Our data show that cavin-1-structured caveolae control cell shape parameters, namely, volume and spreading, i.e., the height of cells. The TEM opening speed and theoretical modelling further suggest a role for caveolae in regulating membrane tension. Interestingly, we provide compelling evidence showing that, in contrast to cavin-1 structured caveolae, non-caveolar caveolin-1 contributes to membrane rigidity, accounting for the limitation on maximal TEM width.

## Results

### Loss of caveolin-1 sensitizes mice to EDIN-B lethal effects during *S. aureus* sepsis

No evidence has been reported to show that EDIN factors in *S. aureus* induce lethal effects on mouse models of infection. In this study, we aimed to compare the susceptibility of caveolin-1-deficient mice (CAV1<sup>-/-</sup>) and their wild-type littermates (CAV1<sup>+/+</sup>) to the wild-type *S. aureus* strain LUG1799 (WT *edinB*). LUG1799 belongs to the European lineage ST80 derived from community-acquired methicillin-resistant *S. aureus*, and it expresses the exotoxin EDIN isoform B (EDIN-B) (18). We first monitored the mortality of CAV1<sup>-/-</sup> and CAV1<sup>+/+</sup> mice challenged with three increasing concentrations of *S. aureus* strain WT *edinB* inoculated through 7 days (Fig. 1A). We did not observe any role for caveolin-1 in mice infected with the predicted nonlethal or lethal inoculum levels. Indeed, all the mice, except one CAV1<sup>-/-</sup> mouse, recovered from infection induced with  $5 \times 10^6$  CFU of bacteria/mouse, and all the CAV1<sup>-/-</sup> and CAV1<sup>+/+</sup> mice died 24 hours after intravenous injection of  $5 \times 10^8$  CFU of bacteria/mouse. In contrast, when mice were challenged with an intermediate dose of  $5 \times 10^7$  CFU/mouse, we found significant differences in susceptibility between CAV1<sup>-/-</sup> and CAV1<sup>+/+</sup> mice ( $P < 0.001$ , Mantel–Cox test). All the CAV1<sup>-/-</sup> mice died on day 1 post-challenge, whereas the death of CAV1<sup>+/+</sup> mice was delayed between 1 and 2 days, with one CAV1<sup>+/+</sup> mouse surviving. The loss of caveolin-1 expression thus sensitized the mice to systemic infection by *S. aureus*-produced EDIN-B. We then explained a possible role for EDIN-B in

150 enhancing CAV1<sup>-/-</sup> mouse susceptibility to systemic infection. Despite a broad concentration range  
151 for the 5 inocula, we recorded no difference in the susceptibility of CAV1<sup>+/+</sup> mice to either the *S.*  
152 *aureus* WT *edinB* or *edinB*-deleted isogenic strain ( $\Delta$ *edinB*) (Fig. S1). Nevertheless, a lethal dose  
153 50 (LD<sub>50</sub>) of approximately  $2.5 \times 10^7$  CFU/mouse was established for both strains of *S. aureus* (Fig.  
154 1B). Interestingly, when we performed similar experiments with an inoculum of  $2.5 \times 10^7$   
155 CFU/mouse, we found a significant difference in the survival rates of CAV1<sup>-/-</sup> mice:  $n=11/18$  mice  
156 infected with *S. aureus*  $\Delta$ *edinB* versus  $n=4/17$  mice infected with *S. aureus* WT ( $P = 0.0123$ ,  
157 Mantel–Cox test) (Fig. 1C). Our study of caveolae-deficient mice reveals the capacity of the EDIN-  
158 B exoenzyme to trigger lethal effects in mice via septicemia.

159

### 160 Caveolae control the extent of TEM tunnel formation

161 The cytotoxic effects of EDIN-like factors include the disruption of RhoA-driven stress fibre and  
162 TEM tunnel formation. TEM tunnel formation is triggered by EDIN-like factors and ExoC3 from  
163 *C. botulinum*, which is widely used in cell biology studies (Fig. 2A). We first verified that ExoC3  
164 treatment exerted no impact on caveolin-1 and cavin-1 expression in treated HUVECs (Fig. S2A).  
165 Importantly, the stoichiometry of caveolin-1 and cavin-1 is highly regulated in endothelial cells  
166 (41). In this study, we observed that short interfering RNA (siRNA)-mediated depletion of  
167 caveolin-1 expression (siCAV1) also led to the estimated reduction in cavin-1 expression by  
168 approximately 80%. We also found that the cross-depletion effect triggered by siRNA targeting  
169 cavin-1/PTRF (siPRTF) on a cellular pool of caveolin-1 was less pronounced, with an approximate  
170 60% reduction in caveolin-1 signal in cells treated with siPRTF. (Fig. S2B). Similar outcomes  
171 obtained with siCAV1 and siPRTF indicated the importance of caveolae structures, and different  
172 outcomes indicated specific roles for caveolin-1. We then verified that siRNA-targeted depletion  
173 of caveolin-1 and cavin-1/PTRF did not affect the extent of ADP ribosylation of RhoA (Fig. S2C).  
174 Ultimately, we verified that TEM tunnels were surrounded by an F-actin bundle after both siCAV1  
175 and siPRTF exposure (Fig. 2A). We then quantified the efficiency of TEM tunnel formation in  
176 siCAV1- or siPRTF-treated cells (Fig. 2B). HUVECs were treated with siCTRL, siCAV1 or  
177 siPRTF followed by ExoC3. We recorded a higher percentage of cells with TEMs in the population  
178 of caveolae-deficient cells: 37.6% (s.d. of 1.3%) in the siCAV1-treatment condition, 36.4% (s.d. of  
179 1.3%) in the siPRTF-treatment, compared to 28.8% (s.d. of 1.2%) in the siCTRL group (Fig. 2B).  
180 Moreover, we observed a significant increase in the density of TEM tunnels per cell in both the  
181 siCAV1 ( $N_{\text{TEM}_{\text{siCAV1}}} = 1.96$ ) and siPRTF ( $N_{\text{TEM}_{\text{siPRTF}}} = 1.36$ ) groups, compared to that of the  
182 siCTRL ( $N_{\text{siCTRL}} = 0.91$ ) group (Fig. S3A). Increases in TEM tunnel density per cell and within the  
183 cell population were similar when cells were treated with EDIN but not ExoC3 (Fig. S3B-C). To  
184 further determine the importance of caveolae in counteracting TEM tunnel formation, we depleted  
185 the caveolae-stabilizing protein EHD2 in cells. The depletion of EDH2 in siEHD2-treated cells did  
186 not affect the cellular levels of caveolin-1 and cavin-1 (Fig. S4A). An analysis of the population of  
187 EHD2-depleted cells revealed an increase in TEM tunnel formation as well as an increase in the  
188 density of TEMs per cell (Fig. S4B and S3C). These data show that caveolin-1, cavin-1, and EHD2  
189 hence fully assembled caveolae limit the formation of TEMs in the cell population.

190

### 191 Caveolae control the cell shape parameters of volume and spreading

192 Reduction in cell height once RhoA signalling is inhibited favours the fusion of apposing plasma  
193 membranes, creating TEM nucleation sites (14, 15). The height of cells is proportional to the  
194 volume divided by the cell spreading area. Hence, we investigated the role played by RhoA,  
195 caveolin-1 and cavin-1 in the extent of cell spreading and associated volume variations. The  
196 spreading area was estimated from the measurement of cell perimeters (Fig. 3A). The volume of  
197 cells was quantified via fluorescent dye exclusion, as depicted in Fig. 3B and as previously  
198 described in (42). Upon cell treatment with siCTRL, we recorded a significant increase, of 17%, in  
199 the spreading area and cell volume (Fig. 3C-D, Table 1). Nevertheless, alterations to cell shape

200 resulted in a 15% decrease in cell height (Table 1). When siCAV1- and siPTRF-treated cells were  
201 treated with ExoC3, we recorded an increase in the spreading area of cells, and this effect was  
202 associated with a reduction in the volume of the cells (Fig. 3C-D). Thus, ExoC3 treatment induced  
203 a 20% reduction in cell height after siCAV1 treatment and a 24% reduction after siPTRF treatment  
204 compared to height in the siCTRL-treated cells (Table 1). The magnitude of the cell height  
205 reduction was in good agreement with our findings showing that siCAV1 and siPTRF treatments  
206 promoted TEM formation to a similar extent. There was a noticeably stronger regulatory effect of  
207 siPTRF treatment compared to that of siCAV1 treatment on cell shape parameters, further  
208 indicating the key role of caveolae in the control of the cell shape parameters of volume and  
209 spreading (Fig. 3C-D). In conclusion, the disruption of caveolae in ExoC3-treated cells produced a  
210 concomitant decrease in cell volume and an increase in cell spreading.

211

### 212 **Caveolae and F-actin distribution at the plasma membrane**

213 Although the crosstalk between RhoA and caveolae controls endothelial cell adaptation to  
214 mechanical load, we lack information on whether inhibition of RhoA modulates the density of  
215 caveolae invaginations at the endothelial cell plasma membrane. We implemented a well-  
216 established unroofing approach to expose the plasma membrane and directly visualize caveolae on  
217 a platinum replica via transmission electron microscopy (43). In both naïve and ExoC3-treated  
218 cells, we observed membrane-associated cytoskeleton filaments, the honeycomb structure of  
219 clathrin sheets, clathrin-coated pits, and rough aspects of pits similar to caveolar invaginations (Fig.  
220 4A). However, the density of the cytoskeletal filaments was less pronounced in the ExoC3-treated  
221 cells. Cells were then transfected with a plasmid encoding GFP-caveolin-1 to confirm the presence  
222 of caveolin-1 in caveolae invaginations by GFP-immunogold labelling (Fig. 4B). Notably, we did  
223 not observe a detectable accumulation of caveolae around TEM tunnels. We quantified the density  
224 of caveolae in control and ExoC3-treated cells. In total, we analysed an area  $A_{\text{area}} > 175 \mu\text{m}^2$  under  
225 each condition ( $n \geq 16$  membrane areas of independent cells per condition). We found a 1.6-fold  
226 decrease in the mean density of caveolae in the ExoC3-treated cells, which exhibited 3.4  
227 caveolae/ $\mu\text{m}^2$  of membrane (s.d. of 2.1 caveolae/ $\mu\text{m}^2$ ), compared with 5.4 caveolae/ $\mu\text{m}^2$  of  
228 membrane (s.d. of 3.1 caveolae/ $\mu\text{m}^2$ ) in untreated control cells (Fig. 4C). Since RhoA-mediated  
229 formation of a cytoskeleton tensile meshwork counteracts the formation of TEM tunnels, we  
230 performed 2D stochastic optical reconstruction microscopy (STORM) imaging, which revealed a  
231 thin F-actin meshwork with  $\sim 30$ -nm resolution. Fig. 4D depicts examples of the high density F-  
232 actin meshwork that formed between well-defined thick actin cables in untreated HUVECs. In  
233 contrast, after siRNA treatment, the cells treated with ExoC3 showed profound reorganization of  
234 the actin cytoskeleton, which formed a loose meshwork of thin actin filaments and bundles  
235 intercalated with large zones devoid of F-actin. We found significant widening of the F-actin  
236 network mesh in ExoC3-treated cells (Fig. 4E). Altogether, cells treated with ExoC3 displayed  
237 caveolae invaginations at the membrane and a loosened actin filament mesh.

238

239

240

### 241 **Different impacts of cavin-1 and caveolin-1 on TEM tunnel formation dynamics**

242 Given the large transcellular apertures formed, the density, diameter and lifetime of TEM tunnels  
243 are thought to be critical in the control of endothelial permeability. To quantify the effects of  
244 caveolae and describe them in a biophysical model of their functions, we implemented a pipeline  
245 of live-cell imaging and semiautomatic measurements of TEM opening speed and TEM maximal  
246 width (15). This method relied on the detection of the Lifeact-GFP signal at the edge of TEM  
247 tunnels and postprocessing of variations in TEM tunnel width as a function of time (15). After we  
248 performed this analysis, we projected the surfaces of all the TEM tunnels that had formed over a 1-  
249 hour period of recording when they first opened onto the image showing the Lifeact-GFP signal

(Fig. 5A). Thus, we established that a TEM opened preferentially at the cell periphery where the cell height was minimal and that TEMs showed negligible overlap (Fig. 5A). Notably, we observed wider TEMs in siCAV1-treated cells than in the siPTRF- and siCTRL- treated cells (Fig. 5A). A quantitative analysis established a 5.4-fold increase in the maximal TEM formation area under caveolin-1-depleted conditions compared to that under siPTRF and siCTRL conditions (Fig. 5B). The widening of TEMs in siCAV1 was observed within the same time frames in which TEM opening was initiated ( $t_{\max}$ ):  $t_{\max\_siCTRL}=1.4$  min (s.d. of 2.3 min.),  $t_{\max\_siCAV1}=1.3$  minutes (s.d. of 1.3 min) and  $t_{\max\_siPTRF}=1.2$  min (s.d. of 1.4 min.). In addition, overall similarities in TEM tunnel formation cycles were observed between 13 and 16 min (Fig. 5C). Altogether, these findings point to caveolin-1 as a key regulator of the opening speed and widening of TEMs (Fig. 5C). According to the dewetting theory as applied to TEM opening, the impact of membrane tension was maximal at the onset of TEM opening (see the physical model description in the Materials and Methods). This finding prompted us to obtain a second series of live-cell imaging at a 10-fold higher temporal resolution to resolve the TEM opening speed ( $S_o$ ). In good agreement with our aforementioned data, we recorded no significant variation in the TEM opening speed between the siCTRL- and siPTRF-treated groups (Fig. 5D). In contrast, we recorded a 2-fold higher TEM opening speed in caveolin-1-depleted cells  $S_{o\_siCAV1}=2.4 \mu\text{m}^2/\text{s}$  versus  $S_{o\_siCTRL}=1.1 \mu\text{m}^2/\text{s}$  (Fig. 5D). Thus, caveolin-1 specifically controls the TEM opening speed and maximal width.

We next inferred changes in membrane mechanical properties due to the depletion of caveolin-1 or cavin-1 using a refined theoretical model of cellular dewetting. This model accounts for the presence of several TEMs opening simultaneously (see the physical model description in the Materials and Methods). Importantly, the model is based on the hypothesis that plasma membrane deformation enabling TEM nucleation and tunnel growth is a function of membrane tension and bending rigidity, within the limit of an absence of contribution of membrane adhesion to cortical cytoskeleton (44). Indeed, consistent with a minimal impact of cortical cytoskeleton elements on membrane mechanics, the data recorded via 2D STORM established the disassembly of the F-actin meshwork in ExoC3-treated cells (Fig. 4D). As explained in the Materials and Methods section in detail, the model predicts that the initial opening speed is proportional to the membrane tension, consistent with a critical role of membrane tension in TEM growth (9). From this, the model predicted a significant 2-fold increase in membrane tension in cells treated with siCAV1, and no effect in siPTRF-treated cells. The model also predicted a maximum TEM size that greatly depended on membrane bending rigidity but weakly on initial membrane tension. Thus, by fitting the observed maximum TEM sizes observed under different treatment conditions, the model provided an estimate of the variation in bending rigidity. Using values obtained from the siCTRL-treated cells, which were a  $\sigma_0 = 2.5 \cdot 10^{-5}$  N/m for the initial membrane tension (45) and  $\kappa = 23.4$   $k_B T$  for the bending rigidity (direct measurement, see below), the model predicts a 15% reduction in membrane bending rigidity upon silencing of cavin-1 expression; and a 55% reduction after caveolin-1 depletion (Fig. 5D). In conclusion, the physical model points to the dominant function of caveolin-1 in controlling membrane tension and bending rigidity, which stands in contrast with the marginal or negligible effect predicted for cavin-1.

### 291 **Differential impacts of caveolin-1 and cavin-1 on membrane rigidity**

292 Our study of TEM dynamics suggests that caveolin-1 controls membrane mechanical parameters,  
293 particularly bending rigidity. Therefore, this parameter was further investigated using a quantitative  
294 method based on pulling membrane tethers from cell-derived plasma membrane spheres (PMSs)  
295 (22, 46, 47) (Fig. 6A). The use of PMSs allowed us to study the mechanical properties of the plasma  
296 membrane without the F-actin cortex (47). PMSs prepared from untreated siCTRL-, siCAV1-  
297 treated and siPTRF-treated HUVECs were aspirated into a micropipette, allowing to control the  
298 membrane tension by changing the aspiration pressure. Membrane tethers were pulled from PMSs

299 using optical tweezers which can be used to measure the force,  $f$ , exerted on the membrane tether  
300 at given values of membrane tension (46) (Fig. 6B-C). The following equation shows the scale of  $f$   
301 in relation to the square root of the bending rigidity ( $\kappa$ ) and membrane tension ( $\sigma$ ):

$$f = 2\pi\sqrt{2\kappa\sigma}$$

302 (48); thus, an analysis of the slope of the square of the force as a function of membrane tension  
303 provides a measurement of the membrane bending rigidity of a PMS (Fig. 6D). We measured a  
304 significant decrease of 30% of the bending rigidity from  $\kappa = 23.4$  kBT (s.d. of 0.9 kBT) in siCTRL  
305 conditions to  $\kappa = 17$  kBT (s.d. of 0.9 kBT) in siCAV1 conditions. Moreover, we observed no  
306 significant difference in bending rigidity in siPTRF conditions compared with siCTRL conditions,  
307 with a  $\kappa$  of 23.4 kBT (s.d. of 1.3 kBT) (Fig. 6D). In agreement with the analysis deduced from our  
308 dewetting model, these measurements reveal an intrinsic property of non-caveolar caveolin-1 in the  
309 control of plasma membrane rigidity.

## 311 Discussion

312  
313 We reported the protective function of caveolae against the lethal effect of EDIN-B obtained  
314 from *S. aureus* in mice with septicaemia. Moreover, the cellular depletion of caveolin-1 and cavin-  
315 1, which are essential components of caveolae, as well as the depletion of the ATPase EHD2, an  
316 accessory protein, sensitized endothelial cells to the formation of TEM tunnels. We established a  
317 specific role for caveolin-1 in controlling the initial opening speed and maximal width of TEMs.  
318 These data, framed in the theoretical model of dewetting, led us to infer that caveolin-1 contribute  
319 to membrane rigidity. Moreover, we reported fine-tuning of cell shape by cavin-1-structured  
320 caveolae in response to a decrease in RhoA-driven actomyosin contractility. Cell shape changes  
321 included a decrease of cell volume and an increase of spreading area. Together these effects  
322 decreased the height of the cells and likely favoured the contact of plasma membranes, creating  
323 TEM nucleation sites. In summary, this work establishes new distinct functions for caveolin-1 and  
324 cavin-1-structured caveole in the regulation of membrane rigidity and cell shape, respectively.

325 Considering the analysis of variations in the width of the TEMs using the cellular dewetting  
326 model, we provided compelling evidence that caveolin-1 controls not only the parameters related  
327 to membrane tension but also those related to membrane bending rigidity. This was independently  
328 supported by conducting membrane-tether-pulling experiments using plasma membrane spheres,  
329 where we found that depletion of caveolin-1 decreased membrane rigidity by 30%, whereas cavin-  
330 1 depletion exerted no effect. The differences between analysis of the TEM opening dynamics with  
331 the model that provided a 55% decrease and biophysical measurements can be attributed to several  
332 simplifications used in the theoretical model, notably the simplified treatment of physical  
333 membrane tension variation, cytoskeletal contributions, and interactions between neighbouring  
334 TEM tunnels. The mechanical role played by caveolin-1 is of profound significance considering  
335 that the inclusion of integral proteins into membranes had no impact on bending rigidity of  
336 membranes, as has been reported on the basis of cylindrical bacteriorhodopsin experiments (49),  
337 and the inclusion of integral proteins may even decrease membrane stiffness, as reported for the  
338 conical  $\text{Ca}^{2+}$ -ATPase SERCA (50). Previously performed simulations also confirmed the softening  
339 effect of protein inclusions on membrane shape (51). Nevertheless, our observations can be  
340 explained by a high density of rigidity-promoting inclusions of the plasma membrane ( $\gg 10\%$ ),  
341 which was generally not achievable with the reconstituted membranes described in the  
342 aforementioned references.

343 Caveolin-1 may contribute to plasma membrane rigidification through numerous effects,  
344 i.e., by structuring ordered lipid domains or anchoring the membrane to cortical cytoskeletal  
345 components. Considering this last point, disruption of the interactions between the plasma  
346 membrane and the cell cortex after caveolin-1 depletion is expected to exert little effect upon RhoA  
347 inhibition. This supposition was supported by our STORM images showing dramatic disassembly

348 of the F-actin meshwork in ExoC3-treated cells. Furthermore, the low contribution of cortex–  
349 membrane interactions to membrane mechanics in treated cells was in good agreement with the  
350 decrease in Young’s modulus of the cell cortex in ExoC3-treated cells (14), pointing to dominant  
351 roles for membrane intrinsic mechanical parameters when RhoA is inactivated. Moreover, previous  
352 studies have established that caveolae in the membrane are enriched with cholesterol,  
353 sphingomyelin, and glycosphingolipids, including gangliosides (52, 53), which are known to  
354 rigidify membranes (54). Thus, caveolin-1 might contribute to the enrichment of the plasma  
355 membrane with these lipid species. We did not establish experimental conditions allowing us to  
356 deplete cholesterol without compromising the shape of HUVECs, which precluded proper analysis  
357 of TEM dynamics. Moreover, a previous attempt to increase the width of TEMs by softening the  
358 membrane through the incorporation of poly-unsaturated acyl chains in phospholipids failed, likely  
359 due to the homeostatic adaptation of membrane mechanical properties (15). Further studies are  
360 required to establish whether and how caveolin-1 oligomers (24) can control membrane mechanical  
361 parameters.

362 Our data point towards a major effect of cavin-1 in regulating the spreading area and the  
363 volume of cells, which might be linked to cavin-1 driving caveolae invaginations. Our data showed  
364 that ExoC3 treatment induced an increase in cell volume, and cell spreading was enhanced. With  
365 siCAV1-treated cells, similar effects were observed but only after ExoC3 treatment. The  
366 uncoupling of cell spreading and volume decrease could be due to the known permissive effect of  
367 RhoA signalling; this swelling could induce the establishment of an outwardly rectifying anion  
368 current mediated via the volume-rectifying anion channels (55, 56). Interestingly, a decrease in cell  
369 volume identified upon depletion of cavin-1, and to a lesser extent after caveolin-1 depletion, was  
370 an outcome independent of RhoA signalling. How caveolae control cell volume in treated cells  
371 awaits determination. Our findings suggest that depletion of caveolae, as well as the inactivation of  
372 RhoA, may release membrane-folding mechanical constraints that limit cell spreading. Another  
373 hypothesis is that caveolae control cell shape parameters through cavin-1 regulation of cell  
374 signalling (57–60).

375 The molecular basis of interindividual variability after infection with *S. aureus* is unclear  
376 (39). Here, we established that the loss of caveolin-1 expression sensitized animals to deadly effects  
377 triggered by EDIN-B during *S. aureus* septicaemia. Although we cannot argue for a causal link  
378 between the lethal effect of EDIN-B in caveolin-1-deficient animals and the protective function of  
379 caveolin-1 against TEM tunnel formation in endothelial cells, these findings represent a first step  
380 towards establishing that endothelial cells regulate TEM tunnel formation and width in vivo; this  
381 hypothesis is supported by previous findings showing that inhibition of RhoA reduces paracellular  
382 permeability, increasing transcellular permeability, and that ADP-ribosyltransferases targeting  
383 RhoA increase vascular permeability to induce animal death after TEM opening is no longer  
384 controlled (8, 16). Consistent with the role played by caveolin-1 in infection, analysis of an  
385 observational cohort of patients with *S. aureus* community-acquired pneumonia indicated that  
386 EDIN-B, together with Panton–Valentin leukocytin, is positively associated with the haemoptysis  
387 aetiology, which manifests as blood in sputum (61). In line with this finding, a previous study  
388 suggested that EDIN-B might promote the translocation of *S. aureus* from lung tissues to the  
389 bloodstream (18). Recently, a human genetics study pinpointed the impact of haploinsufficiency of  
390 the OTULIN deubiquitinase in sensitizing host cells to lysis triggered by the highly prevalent  $\alpha$ -  
391 haemolysin pore-forming toxin of *S. aureus*, a major determinant in staphylococcal pneumonia  
392 (39). Patients with a deleterious OTULIN allele suffer life-threatening skin disease and pulmonary  
393 necrosis typically triggered by *S. aureus* infection. Defects in OTULIN activity lead to an increase  
394 in caveolin-1 cellular protein levels in dermal cells, which in turn upregulates the expression of  
395 ADAM10, which encodes a host cell surface receptor of an alpha-haemolysin pore-forming toxin.

396 Collectively, our findings ascribe caveolin-1 expression level variations to key functions in *S.*  
397 *aureus* infection.

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## 402 **Materials and Methods**

403

### 404 **Bacterial strains and culture conditions**

405 *S. aureus* HT20020209-LUG1799, referred to as wild-type WT *edinB* in this study, is a minimally  
406 passaged ST80 *SCCmecIV* PVL+ MRSA strain isolated in France (deposited at  
407 <http://www.ebi.ac.uk/ena>) that is representative of ST80 CA-MRSA clones (62, 63). *S. aureus*  
408 HT20020209-LUG1799 with the *edinB* gene deletion is referred to as  $\Delta$ *edinB* in this study (18).  
409 Both strains were grown in lysogeny broth (LB) with shaking at 200 rpm and 37 °C. Equal growth  
410 kinetics were verified (not shown).

411

### 412 **Mouse infection model**

413 Adult male and female B6.Cg-*Cav1*<sup>tm1Mls</sup>/J mice (strain #: 007083, Jackson Laboratories) and  
414 C57BL/6J mice (Charles River) were housed under specific-pathogen-free conditions at the Institut  
415 Pasteur animal facilities licenced by the French Ministry of Agriculture (B75150102). Mice  
416 received food and water ad libitum, and their weight was recorded daily throughout the study. *S.*  
417 *aureus* strains were cultured in LB at 37 °C until reaching an OD<sub>600</sub> = 1 after overnight culture.  
418 After washing twice in PBS, cell pellets were resuspended in sterile 0.9% NaCl. Infections were  
419 carried out by injecting 300  $\mu$ L serial dilutions of inoculum intravenously into the tail vein of the  
420 mice. Animal survival was monitored daily.

421

### 422 **Ethics statement.**

423 Animal experiments were performed according to Directive 2010/63/EU of the European  
424 Parliament and conformed to the European Council Directive on the approximation of laws,  
425 regulations, and administrative provisions of the Member States regarding the protection of animals  
426 used for experimental and other scientific purposes (86/609/EEC). The experimental procedures  
427 were approved by the Animal Experimentation Ethics Committee and the Risk Prevention Service  
428 of the Institut Pasteur (approval numbers 210119 and N° 21.264) and authorized by the French  
429 Ministry of Higher Education, Research and Innovation (APAFIS #34712-2022011814537622 v2).

430

### 431 **Cell culture, transfection and toxicity**

432 Primary human umbilical vein endothelial cells (HUVECs, PromoCell) were cultured in human  
433 endothelial serum-free medium (SFM, Gibco) containing 20% foetal bovine serum, 20 ng/ml FGF-  
434 2, 10 ng/ml EGF, and 1  $\mu$ g/ml heparin and referred to as complete SFM (SFMc). Cells were cultured  
435 on gelatine-coated culture ware at 37 °C with 5% CO<sub>2</sub> for as many as six passages. For siRNA  
436 transfection, HUVECs were cultured at a density of 38,000 cells/cm<sup>2</sup>. ON-TARGETplus smart pool  
437 siRNA (Dharmacon) targeting human caveolin-1 (L-003467-00-0005), EHD2 (L-016660-00-  
438 0005), cavin-1/PTRF (L-012807-02-0005) or control RNAi SR-CL000 (Eurogentec) was used at  
439 100 nM via magnetofection technology (OZ Biosciences) following the manufacturer's instructions  
440 in serum-free OptiMEM (Gibco). When necessary, cells were electroporated 24 h post-  
441 magnetofection and then used from 48 to 54 h post-magnetofection. HUVEC electroporation of  
442 plasmids encoding caveolin-1-GFP (22) or Lifeact-GFP (Ibidi, GmbH, Planegg/Martinsried,  
443 Germany) was performed as described in (64). Briefly, cells were trypsinized and resuspended in  
444 Ingenio Solution (Mirus) containing plasmid DNA (10  $\mu$ g/10<sup>6</sup> cells) in a 4-mm cuvette  
445 (CellProjects). Cells were then electroporated at 300 V and 450  $\mu$ F with one pulse of a GenePulser

445

446 electroporator (Bio–Rad). Cells were incubated in SFMc, and the medium was replaced 3 h post-  
447 electroporation. Cells were treated 6 h post-electroporation. Recombinant ExoC3 and EDIN were  
448 produced in *E. coli* and purified as described in (8). Cells were treated with ExoC3 or EDIN toxin  
449 at a final concentration of 100  $\mu$ M for 24 h.

### 451 Immunoblotting and Western blotting

452 Proteins were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose  
453 membranes (GE Healthcare). The primary antibodies used were mouse anti-EHD2 (L-05) (Santa  
454 Cruz sc-100724), rabbit anti-Cav1 (Cell Signaling Technology #3238), rabbit anti-PTRF  
455 (Proteintech, 18892-1-AP) and mouse anti-GAPDH (Santa Cruz sc-47724). The secondary  
456 antibodies used were HRP-conjugated anti-mouse or anti-rabbit (Dako). Signals were imaged using  
457 a Fujifilm LAS-3000 system and quantified with ImageJ software.

### 459 Immunofluorescence

460 HUVECs were seeded on a gelatine-coated  $\mu$ -Dish 35 mm, high (Ibidi) and treated as indicated  
461 previously (65). Cells were fixed in ready-to-use paraformaldehyde, 4% in PBS (Bio–Rad).  
462 Immunostaining of fixed cells permeabilized in 0.5% Triton X-100 was performed. FITC-  
463 phalloidin or TRITC-phalloidin at 1  $\mu$ g/ml (Sigma) were used to stain actin, and DAPI (Life  
464 Technologies) was used to label nuclei.

### 466 Video microscopy

467 HUVECs were electroporated with LifeAct-GFP-pCMV as described above and seeded on a  
468 gelatine-coated polymer coverslip dish (Ibidi). After treatment with the toxins (see above), the cells  
469 were supplemented with 25 mM HEPES (pH 7.4) and their proliferation was recorded at 37  $^{\circ}$ C with  
470 a Nikon Ti inverted microscope using an Ultraview spinning disk confocal system (Perkin Elmer).  
471 For determining TEM opening, images were taken every 10 seconds for 1 h. To determine the  
472 opening speed ( $S_o$ ), images were taken every second for 30 min. Acquired videos were analysed  
473 via an ICY-based semiautomatic protocol.

### 475 Cell volume measurement

476 After siRNA treatment and other treatments, cells were seeded onto a PDMS chip ( $2 \cdot 10^6$  cells/mL)  
477 as described previously (42). Briefly, chambers were coated with 10  $\mu$ g/ml fibronectin in PBS (Life  
478 Technologies) for 1 h at RT. Chambers were washed with medium before cell seeding. Cells were  
479 resuspended in medium supplemented with 0.5 mg/ml Alexa Fluor 488 dextran (MW 10 kD; Life  
480 Technologies) and then injected into the chamber. Finally, the chamber was immersed in medium  
481 to prevent evaporation. HUVECs were allowed to adhere for 4 to 6 h in SFMc at 37  $^{\circ}$ C with 5%  
482 CO<sub>2</sub> before acquisition.

483 Images were analysed with MATLAB (MathWorks). The intensity of the background was maximal  
484 in the absence of any object ( $I_{max}$ ) and represented the fluorescence value for the maximal height  
485 of the chamber ( $h_{max}$ ). In contrast, the pillar excludes fluorescence, which therefore reflects the  
486 minimal fluorescence intensity ( $I_{min}$ ). At the cell level, the fluorescence was partially excluded at a  
487 given point for a cell at a given height ( $h_{x,y}$ ). This strategy enables the measurement of the  
488 fluorescence intensity at this point ( $I_{x,y}$ ). After calibrating the fluorescence intensity signal  $\alpha = (I_{max}$   
489  $- I_{min})/h_{max}$ , integration of the fluorescence intensity over the cell area provided the cell volume  
490 ( $V_{cell}$ ):

$$491 \quad V_{cell} = \iint_{x,y} \frac{I_{max} - I_{x,y}}{\alpha} dx dy$$

### 493 Metal replicates and transmission electron microscopy

494 Metal replicates of the ventral plasma membranes of HUVECs cultured on glass coverslips were  
495 obtained by sonication according to a published protocol (Heuser 2000b) and adapted by S.  
496 Vassilopoulos, our collaborator. Briefly, cells were rinsed three times with Ringer's buffer with  
497  $\text{Ca}^{2+}$  and then briefly subjected to a concentration of 0.5 mg/mL poly-L-lysine diluted in  $\text{Ca}^{2+}$ -free  
498 Ringer's buffer (Sigma–Aldrich). Poly-L-lysine was removed by washing with  $\text{Ca}^{2+}$ -free Ringer's  
499 buffer. The coverslips were immersed in KHMgE buffer at 37 °C before sonication (Vibra-Cell  
500 VCX130 ultrasonic processor, Sonics) at a 20% amplitude. The unroofed cell membranes were then  
501 immediately fixed for 30 minutes with 2% glutaraldehyde/2% paraformaldehyde (PFA). The cell  
502 membranes were sequentially treated with 1%  $\text{OsO}_4$ , 1.5% tannic acid and 1% uranyl acetate before  
503 dehydration via successive ethanol baths, which was ultimately substituted with  
504 hexamethyldisilazane (HMDS) (#C16700-250; LFG Distribution).  
505 For immunogold labelling, sonicated plasma membranes were fixed with 4% PFA before  
506 incubation with primary (GFP Thermo Fisher A11122 Rabbit 1/20) and secondary antibodies  
507 coupled to gold beads (rabbit-gold 815.011 AURION goat 1/25), and the membranes were then  
508 incubated with a  $\text{NaBH}_4$  solution to inactivate aldehydes. The membranes were finally fixed with  
509 2% glutaraldehyde and subjected to the same treatment as that used for morphology studies. The  
510 dehydrated samples were metalized via rotary metallization. The coverslips were placed in the  
511 chamber of a metallizer (ACE600, Leica Microsystems). Once under a high vacuum ( $>10^{-5}$  mBar),  
512 the membranes were covered with 2 nm of platinum stabilized by 4 to 6 nm of braided carbon. The  
513 resulting platinum replicas were separated from the glass by flotation on acid, washed several times  
514 in distilled water baths containing 0.1% detergent (one drop in 10 mL, Photo-Flo, Kodak), and  
515 placed on electron microscopy (EM) grids covered with a carbon film (200 mesh formvar/carbon,  
516 LFG Distribution). The grids were mounted in the goniometer with eucentric side entry of a  
517 transmission electron microscope operating at 80 kV (CM120, Philips), and images were recorded  
518 with a Morada digital camera (Olympus). The images were processed by ImageJ software to adjust  
519 brightness and contrast and are presented in reverse contrast.

520

### 521 **RhoA ADP-ribosylation assay**

522 HUVECs were seeded at a density of 27,000 cells/cm<sup>2</sup> transfected with siCTRL, siCAV1 and  
523 siPTRF and then treated with or without ExoC3. Cells were lysed in ADP-ribosylation buffer (20  
524 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 5 mM  $\text{MgCl}_2$  and cComplete protease inhibitor EDTA-  
525 free (Roche), pH 7.5) and passed through a 27G syringe 20 times. Cell lysates were collected by  
526 centrifugation at 12,000 g for 10 min, and the protein concentration was determined by BCA assay  
527 (Thermo Fisher Scientific). The reaction was carried out by incubating 20  $\mu\text{g}$  of cell lysate with 2  
528  $\mu\text{g}$  of ExoC3 and 10  $\mu\text{M}$  6-biotin-17-NAD<sup>+</sup> (BioLog) at 37 °C for 30 min. The reaction was  
529 terminated by the addition of 1 mM DTT and Laemmli buffer (0.3 M Tris-HCl, 10% SDS, 37.5%  
530 glycerol and 0.4 mM bromophenol blue) and boiling at 100 °C for 5 min. The samples were  
531 subjected to 12% SDS–PAGE, and the proportion of ADP-ribosylated (i.e., biotin-ADPr-RhoA)  
532 RhoA in the sample was measured by Western blotting using streptavidin–peroxidase.

533

### 534 **Plasma membrane sphere formation and tether extraction**

535 Plasma membrane spheres (PMS) were generated via a protocol adapted from (47). Cells were  
536 grown to 60–80% confluence on gelatine-coated 100-mm dishes and incubated for 6–8 h in PMS  
537 buffer [1.5 mM  $\text{CaCl}_2$ , 1.5 mM  $\text{MgCl}_2$ , 5 mM HEPES, 2 mg/ml glucose, 150 mM NaCl, 10  $\mu\text{M}$   
538 MG132 in PBS (pH 7.4)] to induce membrane swelling of the PMSs. Individual PMSs were  
539 aspirated using a casein-passivated micropipette connected to a piezo-stage (PI, Karlsruhe,  
540 Germany) for manipulation and to a hydrostatic aspiration control system (66). Micropipettes were  
541 made in house with borosilicate capillaries pulled into fine cones using a laser pipette puller (P-  
542 2000, Sutter Instrument Co.) and microforged at the desired inner diameter (3–4  $\mu\text{m}$ ) as described  
543 previously (22). For the extraction of tethers from PMSs, we used optical tweezers built in-house

544 that consisted of a single fixed laser beam (infrared laser wavelength of 1070 nm) focused through  
545 a 60X water objective mounted on a confocal microscope (Nikon TE2000 inverted microscope)  
546 (67). To pull tethers from PMSs, we coated streptavidin beads (3  $\mu\text{m}$  in diameter, Spherotech) with  
547 fibronectin, which allowed the beads to bind to PMSs. A membrane tether was generated by  
548 bringing the micropipette-held PMS into contact with a bead trapped by the optical tweezers, and  
549 then by moving the PMS away from the bead. After extraction, the tether was held at a constant  
550 length between 2 and 5  $\mu\text{m}$ , and tether forces were measured during gradual increase in aspiration  
551 pressure and thus PMS tension. At a given membrane tension, the corresponding tether force was  
552 measured at least 30 s after the pressure change and when equilibration was established. Analysis  
553 was performed as described below. Briefly, for each membrane tether, the tether force ( $f$ ) was  
554 plotted as a function of the square root of the membrane tension ( $\sqrt{\sigma}$ ) calculated using the Laplace  
555 law (68) and corrected when the length of the “tongue” of a PMS tether inside the micropipette was  
556 shorter than the radius of the pipette (69). For each tether, we determined a force  $f_0$  corresponding  
557 to the intercept of the linear regression ( $f$  vs.  $\sqrt{\sigma}$ ). To estimate the bending rigidity for PMSs  
558 obtained from membranes of cell treated differently (siCTRL, siCAV1 and siPTRF cells), the data  
559 obtained for all tethers were pooled and rescaled as  $(f-f_0)^2/8\pi^2$  and plotted as a function of membrane  
560 tension. The corresponding PMS rigidity was obtained from the slope of the linear fit.

561

### 562 **Single-Molecule Localization Microscopy of F-actin**

563 For single-molecule localization microscopy (SMLM) of F-actin, we used 2D STORM. HUVECs  
564 were cultured on 1.5 high-performance coverslips coated with 10  $\mu\text{g}/\text{mL}$  fibronectin for 2 h at room  
565 temperature. Soluble G-actin was pre-extracted (0.25% Triton, 0.1% glutaraldehyde in 80 mM  
566 PIPES, 5 mM EGTA, and 2 mM  $\text{MgCl}_2$ , pH 6.8) for 30 s at 37  $^\circ\text{C}$ . The cells were then treated with  
567 glutaraldehyde (0.25% Triton, 0.5% glutaraldehyde in 80 mM PIPES, 5 mM EGTA, 2 mM  $\text{MgCl}_2$ ,  
568 pH 6.8) for 10 min at 37  $^\circ\text{C}$ . Glutaraldehyde was quenched for 7 min at RT (0.1%  $\text{NaBH}_4$  in PBS).  
569 Saturation was reached after 1 h at RT (0.22% gelatine and 0.1% Triton X-100 in PBS, pH 7.3).  
570 Actin filaments were stained overnight at 4  $^\circ\text{C}$  with phalloidin-AF647 (500 nM in PBS). Cells were  
571 then placed in switching buffer freshly prepared before imaging [50 mM Tris, 10 mM NaCl, 10%  
572 glucose, 0.5 mg/mL glucose oxidase, 50  $\mu\text{g}/\text{mL}$  catalase, and 50-100 mM cysteamine (MEA, pH  
573 8.3)] in the presence of phalloidin A647 (10-50 nM). Super-resolved images were acquired with an  
574 Elyra-7 SMLM microscope (Carl Zeiss, Germany) using a  $\text{na}=1.4$  oil 63x objective (Carl Zeiss,  
575 Germany) with 1.518 refractive index oil (Carl Zeiss, Germany) and a pco.edge 4.2 camera system  
576 (Excelitas PCO GmbH). All processing analyses were performed with Zen software (Carl Zeiss,  
577 Germany). Localization of individual molecules was determined using a peak mask size of 15-pixel  
578 diameter and a peak intensity-to-noise ratio between 5 and 6. Drift was corrected using a model-  
579 based algorithm from Zen software. Super-resolution images were reconstructed at a 20-nm pixel  
580 size.

581

582

### 583 **Mesh analysis of the F-actin network imaged via 2D STORM**

584 To quantify actin mesh size from the superresolution images, we developed an image analysis  
585 workflow to reliably segment the delimiting continuous actin filaments. First, we clipped the  
586 images to a maximum detection count of 10 and applied a line enhancement filter to suppress short  
587 apparent discontinuities by modifying a previously described approach (70). Specifically, the  
588 maxima of the mean intensities along line profiles at 15 equally spaced orientations around each  
589 pixel were calculated (line length 21 px or approximately 0.4  $\mu\text{m}$ ). Subsequently, tube-like  
590 structures were extracted from the line-enhanced images by applying a Hessian filter implemented  
591 in scikit-image (smoothing width of 2 px) as described previously (71) and binarising the result  
592 using Otsu thresholding. The obtained masks were subdivided into putative filament segments by  
593 defining sections of equal length along the mask's morphological skeleton and assigning each pixel

594 to its nearest segment (the chosen length was 20 px or approximately 0.4  $\mu\text{m}$ ). The final actin  
 595 filament segmentation was performed considering only segments that: 1) were not morphologically  
 596 connected to at least one other segment, to exclude non-filament detections, or 2) exhibited a mean  
 597 detection count lower than the super-resolution image Otsu threshold. Finally, the actin meshwork  
 598 was obtained as the connected components of the inverted actin filament segmentation mask.

## 600 Physical modelling

601 TEM dynamics were theoretically interpreted on the basis of the generalization of our earlier model,  
 602 which was used for a single TEM (21, 65), to account for the case when several TEMs open  
 603 simultaneously. This generalization was previously used to interpret the data in (15), and we present  
 604 it here for completeness. The opening of a TEM is driven by a net force,

$$F_d = 2\sigma - \frac{T}{R}, \quad (1)$$

605 where  $\sigma$  is the membrane tension,  $T$  is the line tension, and  $R$  is the TEM radius. For a model lipid  
 606 membrane in the entropic regime, membrane tension  $\sigma$  depends on  $R$ , quantified by the Helfrich's  
 607 law. Written in a generalized form to describe  $N$  simultaneous TEMs in the same cell, Helfrich's  
 608 law states that:

$$\sigma = \sigma_0 \exp \left[ -\frac{\sum_{i=1}^N R_i^2}{R_c^2} \right] \quad (2)$$

609 where  $R_c^2 = (R_{cell}^2 k_B \hat{T}) / (8\pi\kappa)$ ,  $R_{cell}$  is the radius of the cell,  $k_B$  is the Boltzmann constant,  $\hat{T}$  is the  
 610 temperature, and  $\kappa$  is the effective bending rigidity of the cell membrane. While rigorously derived  
 611 for a pure lipid membrane, we assumed that Helfrich's law is applicable to describe the relationship  
 612 between the effective membrane tension  $\sigma$  acting on TEMs and the observed projected surface in  
 613 our cells. Thus, the parameter  $\kappa$  in Eq. 2 is an *effective* bending rigidity that accounts for the role  
 614 played by protein inclusions and the mechanical contribution of the remaining cytoskeletal elements  
 615 after cell treatment with the toxin. Moreover, we assumed that an eventual buffering role played by  
 616 the remaining caveolae can be described through modification of the effective parameters  $\sigma_0$  and  
 617  $\kappa$ .

618 As discussed by (21), the effective line tension is not a constant; it increases with time due to the  
 619 formation of an actomyosin cable around the TEM. Stefani et al. described this increase as linear,  
 620  $T \sim at$ . The dynamics of TEM opening are derived from a balance between the driving force (Eq. 2)  
 621 and cell-substrate friction. Assuming  $N$  identical TEMs, this balance is

$$2\sigma_0 R \exp \left( -\frac{NR^2}{R_c^2} \right) - at = \mu R^2 \frac{dR}{dt}, \quad (3)$$

622 where  $\mu$  is the friction coefficient. While this equation can be solved numerically, the following  
 623 analytical approximation can also be obtained, as derived in (15):

$$x \exp x^2 \approx \frac{8 \sigma_0^2 \sqrt{N}}{\mu \alpha R_c}, \quad (4)$$

624 where  $x = N^{1/2} R_{max}/R_c$  and  $R_{max}$  are the maximum TEM radii. As discussed in (15), for biologically  
 625 relevant values of the model parameters, Eq. 4 leads to the following approximate estimate of the  
 626 effective membrane bending rigidity:

$$\kappa \approx \frac{k_B \hat{T}}{8\pi} \frac{A_{cell}}{N A_{max}}. \quad (5)$$

627 Considering the experimental results shown in Table 2 and using the estimate  $\kappa \sim (N \cdot A_{max})^{-1}$  based  
 628 on Eq. 5, we deduced an effective bending rigidity after silencing cavin-1/PTRF. These

629 approximate estimates can be refined using Eq. 4 directly, which also takes into account variations  
 630 in membrane tension. The following expression for the bending rigidity is thus obtained:  
 631

$$\kappa \approx \frac{k_B \hat{T}}{8\pi} \frac{R_{\text{cell}}^2}{N R_{\text{max}}^2} \ln \left( \frac{8 \sigma_0^2}{\mu \alpha R_{\text{max}}} \right). \quad (6)$$

632 Eq. 6 is used to obtain the predictions of bending rigidity variations mentioned in the text: a  
 633 reduction in effective bending rigidity by a factor of approximately 1.2 for siPTRF cell membranes  
 634 and by a factor of approximately 2.3 for siCAV1 cell membranes, which is equivalent to a 15%  
 635 decrease for siPTRF and 55% for siCAV1 cell membranes.  
 636

### 637 *Fit of the initial opening speed*

638 For short time intervals, the differential equation for the opening dynamics can be simplified to

$$\mu R^2 \frac{dR}{dt} = 2\sigma_0 \left( 1 - \frac{R_n}{R} \right), \quad (7)$$

639 where  $R_n = T/(2\sigma_0)$  is the minimal nucleation radius. This equation can be integrated to obtain

$$R_n^2 \ln \left( \frac{R}{R_n} - 1 \right) + R_n R + \frac{R^2}{2} - C = \frac{2\sigma_0}{\mu} t, \quad (8)$$

640 where  $C$  is an integration constant, whose value is such that  $R = R_0$  for  $t = 0$ , with  $R_0$  the unknown  
 641 nucleated TEM radius, which is larger than the minimal nucleation radius  $R_n$ ;  $R_0 > R_n$ . Because all  
 642 TEM measurements are reflect  $R \gg R_0 > R_n$ , the dominant term on the far left side is the term  
 643 proportional to  $R^2 = A/\pi$ , where  $A$  is the TEM area. These considerations yield the following  
 644 estimate of  $\sigma_0$ :

$$\frac{\sigma_0}{\mu} = \frac{A_2 - A_1}{4\pi\Delta t}, \quad (9)$$

645 where  $\Delta t = 1$  s is the time interval between two acquisitions;  $A_1$  is the TEM area in the first image  
 646 after TEM opening (taken on average at a time  $\Delta t/2$ ), and  $A_2$  is the TEM area in the second image.  
 647

Condition	$\sigma_0$ ( $\mu\text{N/m}$ )	$A_{\text{max}}$ ( $\mu\text{m}^2$ )	$N$ (average)	$(N \cdot A_{\text{max}})^{-1}$ ( $\mu\text{m}^{-2}$ )
Control	$25 \pm 10$	$8.1 \pm 0.5$	$0.90 \pm 0.09$	$0.137 \pm 0.022$
siCAV1	$50 \pm 7$	$23 \pm 4$	$1.92 \pm 0.15$	$0.023 \pm 0.006$
siPTRF	$29 \pm 7$	$9.3 \pm 0.7$	$1.34 \pm 0.11$	$0.080 \pm 0.012$

648

649 **Table 2.** Estimate of the variation of mechanical cell parameters between different experimental  
 650 conditions. The value of the effective membrane tension  $\sigma_0$  for the control case is obtained from  
 651 earlier estimates (65). The increase in  $\sigma_0$  for siCAV1 and siPTRF cell membranes is deduced from  
 652 our experimental data using Eq. 9. The TEM maximum area,  $A_{\text{max}}$ , is the median not the average  
 653 value because the median is a more robust estimator in the presence of a few extremely large values.  
 654 As discussed in the text, the variations in the bending rigidity are roughly proportional to variations  
 655 in  $(N \cdot A_{\text{max}})^{-1}$ , where  $N$  is the average number of TEMs opening simultaneously and  $A_{\text{max}}$  is the  
 656 TEM maximum area.

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## Statistical analysis

Statistical tests were performed using the R Software v4.2.1(R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>), along with packages lme4 (1.1-30)(72), lmerTest (v3.1-3)(73), emmeans (v1.8.0) (<https://CRAN.R-project.org/package=emmeans>), except for the F-actin mesh size where the Prism Software was used. All experiments were repeated at least three times to ensure reproducibility and corresponding variability was accounted for in statistical analyses using mixed-effect regressions, with a random intercept estimated for every technical replicate. In particular, Binomial outcomes (e.g. presence/absence) were analysed using mixed-effect logistic regression. Continuous measurements (e.g. TEM area) were investigated with a Gamma log-link function in a mixed-effect framework. Contrasts between multiple pairs of categorical predictors were calculated through estimated marginal means with Tukey's correction. P-values were calculated as indicated in the respective figure legends. P-values were considered statistically significant at  $P \leq 0.05$ . Significance levels are indicated as follows: ns: not significant if  $P > 0.05$ ; \* :  $P \leq 0.05$ ; \*\*:  $P \leq 0.01$ ; \*\*\*:  $P \leq 0.001$ ; \*\*\*\*:  $\leq 0.0001$ .

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## H2: Supplementary Materials

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- Fig. S1. CAV1<sup>+/+</sup> mice susceptibility to EDIN-B in Staphylococcal septicaemia  
Fig. S2. Controls of siRNAs and RhoA ADP-ribosylation efficacies.  
Fig. S3. Caveolin-1 and cavin-1/PTRF depletion increases TEM formation in both ExoC3- and EDIN-treated cells.  
Fig. S4. Depletion of the caveolar accessory component EHD2 increases the TEM tunnel formation  
Fig. S5. Caveolin-1 controls the TEM opening speed and maximum size.

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## References and Notes

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931  
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933  
934 **Author contributions:** Animal experiments were performed by M-A.N. and C.M.; cell  
935 biology experiments were performed by C.M.; biochemistry experiments were performed  
936 by C.S., M.M. and C.M.; the physical model was established by D.G-R. All statistical  
937 analyses were performed by C.M. and T.O.; cell volume measurements were performed by  
938 C.M. and N.S. together with M.P.; transmission electron microscopy was performed by  
939 C.M. and E.L. together with S.V. Super-resolution imaging was performed by C.M., A.S.,  
940 and C.Le.; bending rigidity measurements were performed by C.M. and F.C-T. together  
941 with P.B.; video and image analyses were performed by C.M., C.Le. and M.A.; E. Lemichez  
942 and C.Le. designed the experiments and supervised the project. E. Lemichez, C.Le., D. G-  
943 R, C.M., C. La and P.B. wrote the manuscript. All authors reviewed the manuscript.

944  
945 **Competing interests:** The authors declare no competing interest.

946  
947 **Data and materials availability:** All data needed to evaluate the conclusions in the paper  
948 are presented in the paper and/or the Supplementary Materials.

## 951 FIGURES AND TABLES

952

953

**Fig. 1. Susceptibility of CAV1-deficient mice to EDIN-B exoenzyme in *S. aureus* bacteremia.**

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(A-C) Kaplan–Meier survival curves over 7 days for groups of CAV1<sup>-/-</sup> mice and CAV1<sup>+/+</sup> littermates infected intravenously at Day 0 with different doses, expressed as colony-forming units per mouse (CFU/m), of LUG1799 wild-type *S. aureus*, which is positive for *edinB* (WT *edinB*), and the isogenic strain LUG1840 I, in which the *edinB*-encoding gene ( $\Delta$ *edinB*) is deleted. (A) Comparative analysis of CAV1<sup>-/-</sup> and CAV1<sup>+/+</sup> mouse susceptibility to *S. aureus* bacteraemia. Groups of CAV1<sup>-/-</sup> and CAV1<sup>+/+</sup> mice were challenged via intravenous injection of 5×10<sup>6</sup> CFU/mouse ( $n=10$  CAV1<sup>+/+</sup> mice vs.  $n=9$  CAV1<sup>-/-</sup> mice), 5×10<sup>7</sup> CFU/mouse ( $n=7$  CAV1<sup>+/+</sup> mice vs.  $n=9$  CAV1<sup>-/-</sup> mice) or 5×10<sup>8</sup> CFU/mouse ( $n=7$  CAV1<sup>+/+</sup> mice vs.  $n=8$  CAV1<sup>-/-</sup> mice). After administering a dose of 5×10<sup>7</sup> CFU/mouse, we recorded a significant increase in CAV1<sup>-/-</sup> susceptibility to infection compared with that of the CAV1<sup>+/+</sup> littermates. Log-rank test (Mantel–Cox),  $P < 0.0011$  at 5×10<sup>7</sup> CFU/mouse (1 experiment). (B) Lethal dose 50 (LD<sub>50</sub>) of WT *edinB* or  $\Delta$ *edinB* strains administered to mice. Mice were injected with 2.5×10<sup>7</sup> CFU/mouse ( $n=12$  mice infected with either WT *edinB* or  $\Delta$  *edinB* strains, 2 experiments). There were no significant difference in the survival kinetics between WT *edinB*- and  $\Delta$ *edinB*-infected animals, as determined by log-rank test (Mantel–Cox). (C) Comparative analysis of the susceptibility of CAV1<sup>-/-</sup> mice to bloodstream infection triggered by either the WT or  $\Delta$ *edinB* strains. Mice were injected with 2.5×10<sup>7</sup> CFU/mouse ( $n=17$  and 18 mice infected with WT and  $\Delta$ *edinB* strains, from 2 experiments). Log-rank test (Mantel–Cox) showed a significantly higher susceptibility of CAV1<sup>-/-</sup> mice to WT *edinB* than to  $\Delta$ *edinB* ( $P=0.0123$ ).

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**Fig. 2. High number of TEMs in caveolin-1- or cavin-1/PTRF-depleted cells.**

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(A) Confocal spinning disk images showing the actin cytoskeleton of HUVECs treated with an siRNA control (siCTRL) and siRNA against caveolin-1 (siCAV1) and cavin-1 (siPTRF) and left untreated (Control) or treated for 24 h with C3 exoenzyme (ExoC3). Cells were stained with phalloidin-TRITC. Arrowheads show the presence of stress fibres in the absence of ExoC3 treatment. Stars show examples of TEM tunnels surrounded by thick actin cables. Scale bar, 20  $\mu$ m. (B) Histograms show the percentages of ExoC3-treated cells displaying at least one TEM ( $n=1,400$  cells per condition in 8 independent experiments). Graph shows technical replicates pooled together. Error bars show normal asymptotic 95% confidence intervals (CI). Data analysis with mixed-effect logistic regression model (random intercept capturing technical variability) with correction for multiple comparisons using a Tukey’s HSD test. \*\*\*\* $P < 0.0001$ , for both siCTRL vs siCAV1 and siCTRL vs siPTRF conditions. No difference was recorded between siCAV1 and siPTRF conditions.

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**Fig. 3. Regulation of HUVEC area and volume by cavin-1/PTRF, caveolin-1 and RhoA.**

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(A) Confocal spinning disk images of HUVECs stained with phalloidin-FITC and selected perimeters (yellow line) in the absence (- ExoC3) or presence of ExoC3 (+ ExoC3). Scale bar, 20  $\mu$ m. (B) Schematic representation of the device used to define the volume of a cell by measuring the intensity of fluorescence exclusion. Briefly, from the top to the bottom, i) side view of the chamber in which a cell adheres to a coverslip. The PDMS pillar sustains the ceiling (grey), and the maximal height of the chamber  $h_{max}$  (background) is known. The siCTRL-, siCAV1- or siPTRF-transfected HUVECs were seeded in the chamber and remained either untreated or treated with ExoC3. High molecular weight dextran-FITC (green) was added to the chamber being and was excluded from cells (values  $h_{x,y}$ ). ii) Raw epifluorescence image showing a typical field of HUVECs

998 and iii) the graph of fluorescence intensities (in greyscale) which show the function of distance  
999 along the dotted line. Parameters  $I_{max}$  and  $I_{min}$  yield values of maximum and minimum fluorescence  
1000 intensities. Values for cell volume ( $V_{cell}$ ) were obtained by integrating the fluorescence intensities  
1001  $h_{max} - h_{x,y}$  over the cell area. (C) Boxplots show the distribution of values of areas with TEMs as  
1002 estimated from measures of their perimeters, which is shown in (a). Measurements were performed  
1003 with HUVECs transfected with siCTRL, siCAV1 or siPTRF and then treated with ExoC3 (+  
1004 ExoC3) or untreated (- ExoC3). Measurements were performed with  $n>698$  untreated cells and  
1005  $n>595$  treated cells, 5 independent experiments. (D) Boxplots show the distribution of cell volumes,  
1006 as described in (c). Measurements were performed on HUVECs transfected with siCTRL, siCAV1  
1007 or siPTRF and then treated with ExoC3 (+ ExoC3) or untreated (- ExoC3). Data are from  $n=216$   
1008 and  $n=308$  cells after siCTRL  $\pm$  ExoC3 treatment,  $n=197$  and  $n=266$  cells after siCAV1  $\pm$  ExoC3  
1009 treatment and  $n=152$  and  $n=157$  cells after siPTRF  $\pm$  ExoC3 treatment; 3 independent experiments.  
1010 (B-D). Graph shows technical replicates pooled together. The data were analysed with a mixed-  
1011 effect generalized linear model with Gamma log-link function, random intercept accounting for  
1012 technical variability and Tukey's correction for pairwise comparisons between control and each  
1013 siRNA treatment, \*\*\*\* $P<0.0001$ , \*\* $P<0.01$ , \* $P<0.05$  and ns, not significant.  
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1015 **Fig. 4. Inhibition of RhoA decreases caveolae density on the plasma membrane and loosens**  
1016 **the F-actin meshwork.**

1017 (A-B) Transmission electron micrographs show unroofed HUVECs that were either untreated (-  
1018 ExoC3) or treated with ExoC3 (+ ExoC3); caveolae on the ventral side of the plasma membrane  
1019 are evident (A) and anti-GFP immunogold-labelled GFP-caveolin-1 is shown (B). Right panels  
1020 show membrane areas at high magnification, revealing i) caveolae (plain yellow arrowhead), ii)  
1021 clathrin-coated pits and patches (plain white arrowhead), iii) actin filaments (empty white  
1022 arrowhead) and TEM tunnels in treated cells (white star). (B) Immunogold labelled GFP-CAV1 as  
1023 shown in (A). Gold beads are represented by yellow circles. (A-B) Scale bars: control, 1  $\mu\text{m}$ ;  
1024 high magnification, 200 nm; ExoC3, 5  $\mu\text{m}$ ; high magnification, 200 nm. (C) Boxplot shows the density  
1025 of caveolae per  $\mu\text{m}^2$  of the plasma membrane in control versus ExoC3-treated HUVEC.  
1026 Quantification was performed on transmission electron micrographs, see examples in (A). Graph  
1027 shows technical replicates pooled together. Data were analysed with a mixed-effects linear model  
1028 with random intercept and Tukey's correction for pairwise comparison. \* $P=0.027$  ( $n=16$  cells from  
1029 3 technical replicates). (D) 2D STORM images show the disruption of actin bundles and the highly  
1030 dense meshwork consisting of intertwined actin filaments with F-actin in a loose meshwork.  
1031 HUVECs were transfected with siCTRL, siCAV1 or siPTRF and either left untreated or treated  
1032 with ExoC3 before F-actin staining with phalloidin. Scale bar, 10  $\mu\text{m}$ . High magnification images  
1033 are shown in the right panels. Scale bar, 1  $\mu\text{m}$ . (E) Boxplot shows the average mesh size per cell  
1034 ( $\mu\text{m}^2$ ) of the F-actin network. Quantification was performed with 2D STORM images of siCTRL-,  
1035 siCAV1- and siPTRF-treated cells, denoted (+), and ExoC3-treated cells (-).  $P$  values were  
1036 calculated with a nested t test on 8 to 9 cells per group; 3 independent replicates. \*\* $P<0.01$ , \* $P<0.05$   
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1038 **Fig. 5. Caveolin-1 controls the TEM opening speed and maximum size.**

1039 (A) Images show examples of projections of all tunnels upon TEM initial opening (lower panel) in  
1040 HUVECs transfected with Lifeact-GFP expression plasmid and siCTRL, siCAV1 or siPTRF  
1041 captured during 1 h of live imaging. Lifeact-GFP HUVECs transfected with different siRNAs were  
1042 treated with ExoC3 and recorded by live imaging for 1 h. All initial TEM opening was based on  
1043 the first frame in which TEM tunnels formed using ICY. The lower panel shows the projection of  
1044 cumulative areas of initial TEM opening identified during 1 h of live imaging. Scale bars, 20  $\mu\text{m}$ .  
1045 (B) Boxplot shows the distribution of TEMs, the maximal and median area values in HUVECs  
1046 cotransfected with Lifeact-GFP expressing plasmid and siCTRL, siCAV1, or siPTRF prior ExoC3

1047 treatment. Maximal areas were determined on the basis of each kinetic parameter of TEM  
 1048 dynamics, as shown in (C). The data represent  $n > 105$  TEMs in 7 cells of each treatment group;  $> 3$   
 1049 independent experiments. Graph shows technical replicates pooled together. Statistical data  
 1050 analysis using a mixed-effect generalized linear model with Gamma log-link function, random  
 1051 intercept, and Tukey's correction for multiple comparisons. \*\*\*\* $P < 0.0001$ , \*\* $P < 0.01$  and ns, non-  
 1052 significant. (C) The graph shows variations in TEM areas as a function of time expressed in  
 1053 minutes. HUVECs transfected with siCTRL, siCAV1 or siPTRF were treated with ExoC3 for 24 h.  
 1054 The calculated values of  $t_{max}$  that corresponded to the time of opening to the time when the maximal  
 1055 areas were observed and the values of  $t_c$  corresponded to the time frame of a complete cycle of  
 1056 opening and closing are indicated on the graph for each condition. The data are from  $n > 105$  TEMs  
 1057 of 7 cells per treatment;  $> 3$  independent experiments. (D) The graph shows variations in mean  
 1058 values, expressed in seconds, in the TEM areas of cells treated with ExoC3. The curves were plotted  
 1059 with data obtained from time-lapse video recorded at 1 frame/second for 30 min. Lifeact-GFP  
 1060 expressing cells transfected with siCTRL, siCAV1, and siPTRF. The data correspond to  $n > 22$   
 1061 TEMs per condition from 4 independent experiments.  
 1062

1063 **Fig. 6. Caveolin-1 controls membrane bending and rigidity.**

1064 (A) Confocal spinning disk image of a HUVEC displaying a calcein-AM-positive attached plasma  
 1065 membrane sphere (PMS) (arrowhead). Scale bar, 10  $\mu\text{m}$ . (B) Schematic representation of the device  
 1066 used for measuring membrane mechanical parameters. It shows micropipette aspiration (grey) of a  
 1067 PMS (blue) and a tube pulled from the PMS through a bead bound to the PMS and trapped with an  
 1068 optical tweezer (purple). Increasing the aspiration pressure in the pipette allowed a progressive  
 1069 increase in the PMS membrane tension. (C) Confocal images show examples of calcein-AM-  
 1070 positive PMSs prepared from siCTRL-, siCAV1- or siPTRF-transfected cells during micropipette  
 1071 aspiration. Scale bars, 2  $\mu\text{m}$ . (D) the Force required to pull membrane tubes rescaled to  $((f-f_0)^2/8\pi^2)$   
 1072 (in  $\text{pN}^2$ ), which is a function of the membrane tension (mN/m) for different siCTRL, siCAV1 and  
 1073 siPTRF treatments. The force  $f_0$  was measured for each tube as the force when the membrane  
 1074 tension vanishes. The bending rigidity  $\kappa$  (in  $\text{kBt}$ ) was determined via the slope of the linear  
 1075 regression. The data were calculated from  $n=5$  to  $n=10$  tubes per condition ( $> 4$  independent  
 1076 experiments). Linear regression data are shown as dashed lines, and confidence intervals are shown  
 1077 as solid lines (slope  $\pm$  s.d.).  
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1079 **Table 1: Cell spreading area, volume and height.**

Conditions	Mean cell area ( $\mu\text{m}^2$ )	s.d. of the cell area ( $\mu\text{m}^2$ )	Mean cell volume ( $\mu\text{m}^3$ )	s.d. of the cell volume ( $\mu\text{m}^3$ )	Cell height ( $\mu\text{m}$ )	s.d. of the height ( $\mu\text{m}$ )
siCRT-	6,583	5,168	2,320	3,147	0.35	0.75
siCRT+	9,025	7,258	2,710	4,326	0.30	0.72
siCAV1-	7,048	5,583	2,146	3,214	0.30	0.70
siCAV1+	9,831	7,984	2,357	3,010	0.24	0.50
siPTRF-	7,539	5,450	1,914	2,234	0.25	0.48
siPTRF+	10,139	7,873	2,282	2,628	0.23	0.43

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1081 The means and standard deviations (s.ds.) of the cell spreading area (Fig. 3C) and volume (Fig.  
 1082 3D). Cell height was estimated by the ratio between the cell volume and cell area, and the standard  
 1083 deviation was estimated via error propagation.

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## SUPPLEMENTARY MATERIALS

### Fig. S1. CAV1<sup>+/+</sup> mice susceptibility to EDIN-B in Staphylococcal septicaemia.

Kaplan–Meier survival curves with data obtained through 7 days from groups of CAV1<sup>+/+</sup> infected intravenously at Day 0 with different doses of LUG1799 wild-type *S. aureus*, expressed as colony-forming units per mouse (CFU/m). Comparative analysis of CAV1<sup>+/+</sup> mouse susceptibility to bloodstream infection with either the WT *edinB* or  $\Delta$ *edinB* strain. Mice were injected with  $5 \times 10^6$  CFU/m (n=5 mice infected with the WT *edinB* or  $\Delta$ *edinB* strain; 1 experiment),  $10^7$  CFU/m (n=17 and n=19 mice infected with the WT *edinB* or  $\Delta$ *edinB* strain, respectively; 3 experiments),  $5 \times 10^7$  CFU/m (n=19 or n=18 mice infected with the WT *edinB* or  $\Delta$ *edinB* strain; 3 experiments), and  $5 \times 10^8$  CFU/m (n=4 or n=5 mice infected with either the WT or  $\Delta$ *edinB* strain, respectively; 1 experiment). There were no significant differences in the kinetics of survival between the WT and  $\Delta$ *edinB*-infected animals, as determined by log-rank test (Mantel–Cox).

### Fig. S2. Controls of siRNAs and RhoA ADP-ribosylation efficacies.

(A) Immunoblots show caveolin-1 (IB: CAV1) and cavin-1/PTRF (IB: PTRF) in total cellular extracts of HUVECs transfected with siCTRL and then treated for 24 h with ExoC3. Immunoblot GAPDH (IB: GAPDH) was used as the loading control. Immunoblots are representative of 3 independent experiments. (B) Immunoblots of caveolin-1 (IB: CAV1) and cavin-1/PTRF (IB: PTRF) show the specific and cross-depletion effects of siCAV1 and siPTRF. Immunoblot anti-GAPDH antibody (IB: GAPDH) shows equal loading. The graph shows caveolin-1 and cavin-1/PTRF levels normalized to the level of GAPDH (3 independent experiments). (C) Western blots show the fraction of ADP-ribosylated RhoA in cells that were resistant to a second round of in vitro ADP-ribosylation with biotin-NAD<sup>+</sup>. Western blot shows the fraction of ADP-ribosylated RhoA in vitro as detected with streptavidin–peroxidase (IB: Biotin-ADPr) i.e. not modified during cell intoxication. Immunoblotting with an anti-RhoA antibody (IB: RhoA) revealed equal RhoA protein levels. Immunoblotting with an anti-GAPDH antibody revealed equal protein loading. Blots are representative of n=3 independent experiments. The graph shows the mean values of the blot signal intensities  $\pm$  s.ds. from n=3 independent experiments. Values correspond to the signal intensities of biotin-tagged ADP-ribosylated RhoA (Biotin-ADPr) normalized to those of RhoA and GAPDH IB signals. Values are expressed as a percentage compared with the intensity of the siCTRL-transfected untreated cells, which was set to 100%.

### Fig. S3. Caveolin-1 and cavin-1/PTRF depletion increases TEM formation in both ExoC3- and EDIN-treated cells.

(A) The graph shows the distribution of HUVECs between classes defined by TEM tunnel density ranging from a group of cells displaying only 1 TEM and those for which the density increased by a factor of 1 through those cells with 40 TEMs (y-axis). HUVECs were transfected with siCTRL, siCAV1 or siPTRF before treatment with ExoC3. The graph includes the geometric means ( $N_{\text{TEM}}$ ) of the density of TEMs per cell within the whole cell population  $\pm$  s.d. Graph shows technical replicates pooled together. Statistical data analysis using a mixed-effect linear model, with random intercept shows significant differences between values of  $N_{\text{TEM}}$ , \*\*\*\*P<0.0001. Data from n=1,400 cells per condition from 7 independent experiments. (B) Histograms show the percentages of EDIN-treated cells with at least one TEM (n=986 cells per treatment in 5 independent experiments). Error bars show 95% confidence intervals (CIs). Data analysis with a logistic regression model adjusted for multiple comparisons using Tukey’s HSD test, \*\*\*\*P<0.0001; the siCTRL vs. siCAV1 groups and the siCTRL vs. siPTRF groups. No difference was recorded between the siCAV1 and siPTRF

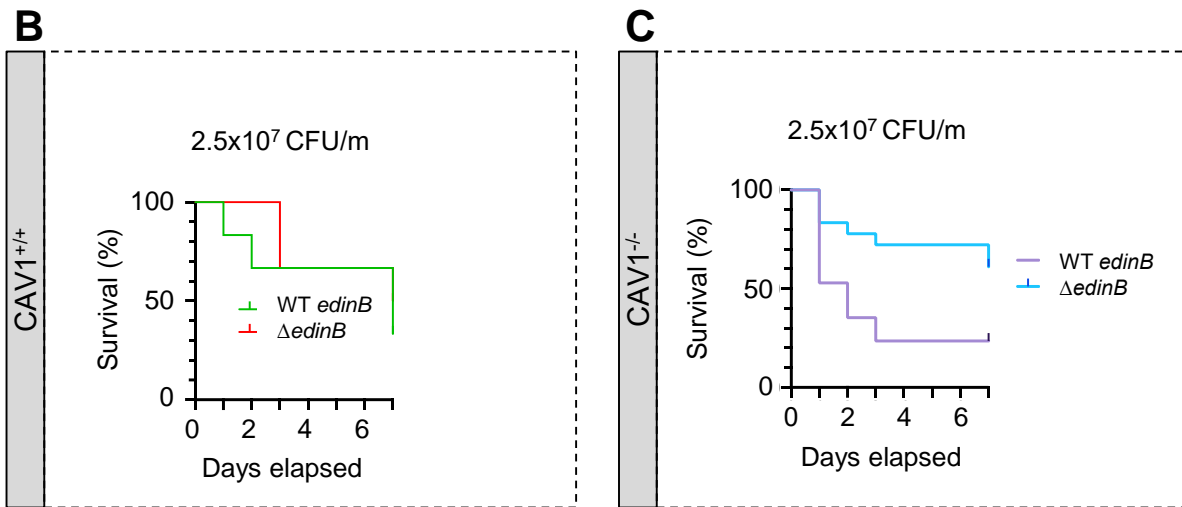
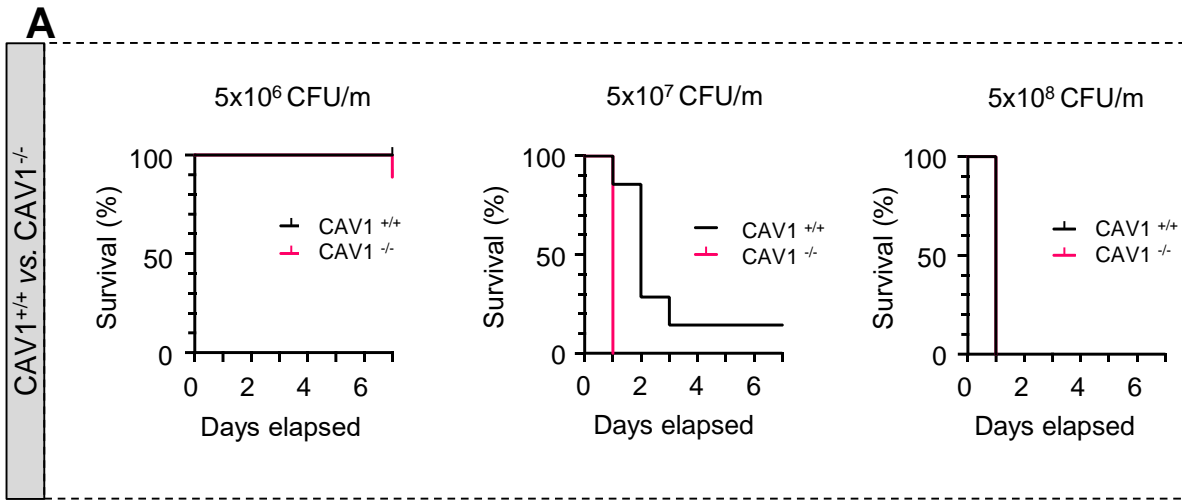
1131 groups. (C) The graph shows the distribution of HUVECs between classes as defined by TEM  
1132 density with ranging from a group of cells displaying only 1 TEM and those for which the density  
1133 increased by a factor of 1 through those cells with 40 TEMs (y-axis). HUVECs were transfected  
1134 with siCTRL, siCAV1 or siPTRF before treatment with ExoC3. The graph includes values the  
1135 geometric means ( $N_{\text{TEM}}$ ) of the density of TEMs per cell within the whole cell population  $\pm$  s.d.  
1136 Statistical data analysis was based on a mixed linear model with a random intercept and fixed effects  
1137 and revealed significant differences between  $N_{\text{TEM}}$  values, \*\*\*\* $P < 0.0001$  ( $n > 986$  cells per  
1138 condition from 5 independent experiments).

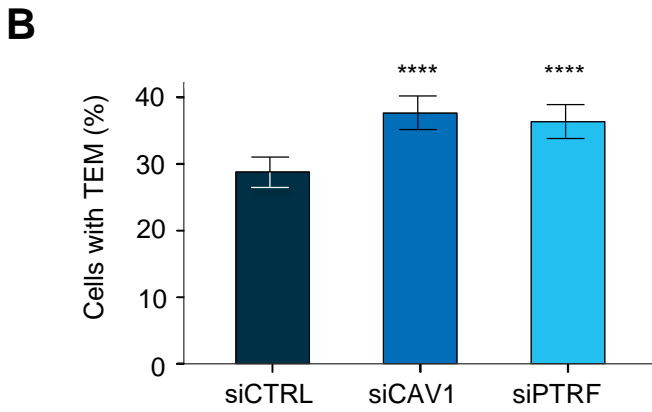
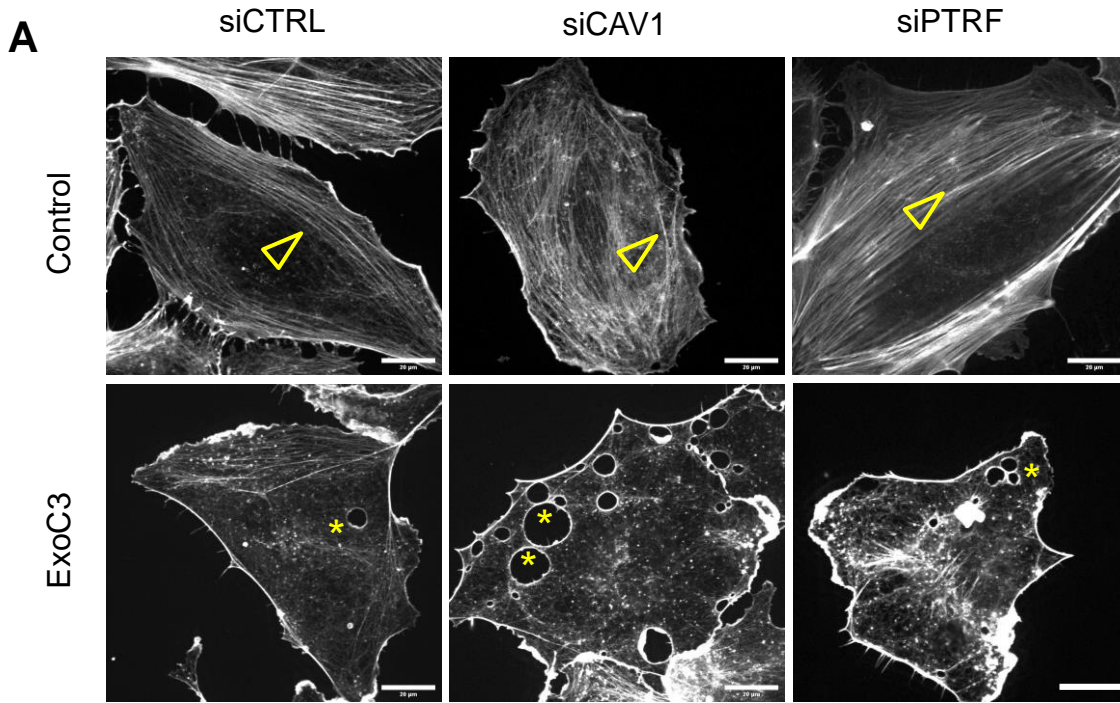
1139  
1140 **Fig. S4. Depletion of the caveolar accessory component EHD2 increases TEM formation.**

1141 (A) Immunoblots show EHD2 cellular levels (IB: EHD2) in total cellular extracts of HUVECs  
1142 transfected with siCTRL or siEHD2 and then treated for 24 h with ExoC3. Immunoblot GAPDH  
1143 (IB: GAPDH) was used as the loading control. Immunoblots are representative of  $n=3$  independent  
1144 experiments. (B) Histograms show the percentages of C3-treated cells displaying at least one TEM  
1145 ( $n=1000$  cells per condition from 5 independent experiments). Error bars show 95% confidence  
1146 intervals (CIs). Data analysis with a logistic regression model adjusted for multiple comparisons  
1147 using Tukey's HSD test, \*\*\*\* $P < 0.0001$  for the siCTRL versus siEHD2 groups. (C) The graph  
1148 shows the distribution of HUVECs between classes as defined by TEM density ranging from a  
1149 group of cells displaying only 1 TEM and those for which the density increased by a factor of 1  
1150 through those cells with 40 TEMs/cell (y-axis). HUVECs were transfected with siCTRL or siEHD2  
1151 before treatment with ExoC3. The graph includes values of the geometric means ( $N_{\text{TEM}}$ ) of the  
1152 density of TEMs per cell within the whole cell population  $\pm$  s.d. Statistical data analysis was based  
1153 on a mixed linear model with a random intercept and fixed effects and revealed significant  
1154 differences between  $N_{\text{TEM}}$  values, \*\*\*\* $P < 0.0001$  ( $n = 1000$  cells per condition from 5 independent  
1155 experiments).

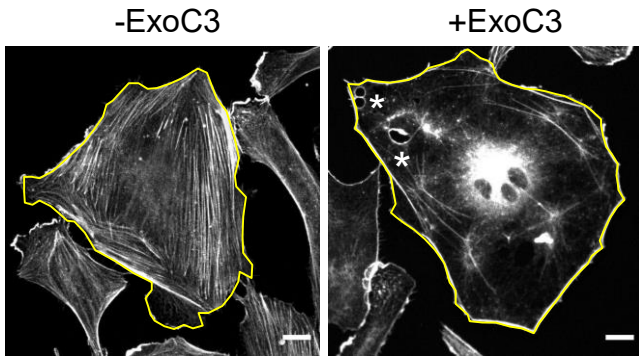
1156  
1157 **Fig. S5. Caveolin-1 controls the TEM opening speed and maximum size.**

1158 The graph shows variations in TEM areas as a function of time expressed in minutes. HUVECs  
1159 transfected with siCTRL, siCAV1 or siPTRF were treated for 24 h with ExoC3. ( $n > 22$  TEMs per  
1160 condition; 4 biological replicates). Experimental data were obtained via a video recorded at 1  
1161 frame/second for 30 minutes.  
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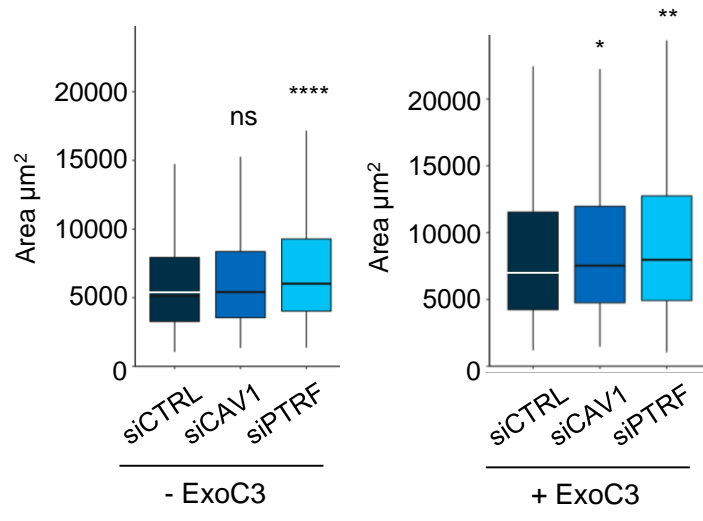




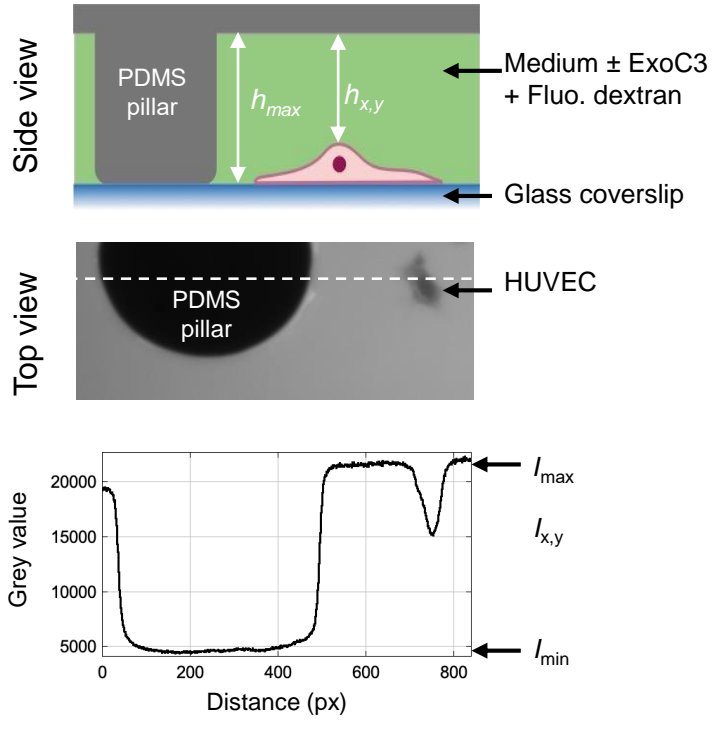
**A**



**C**



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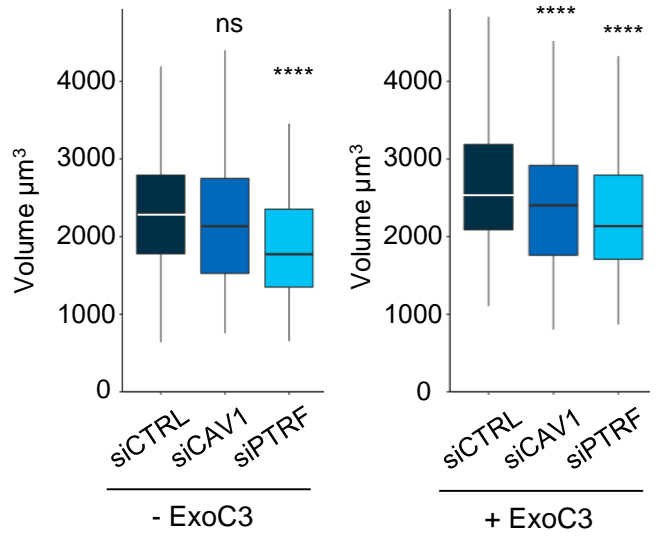
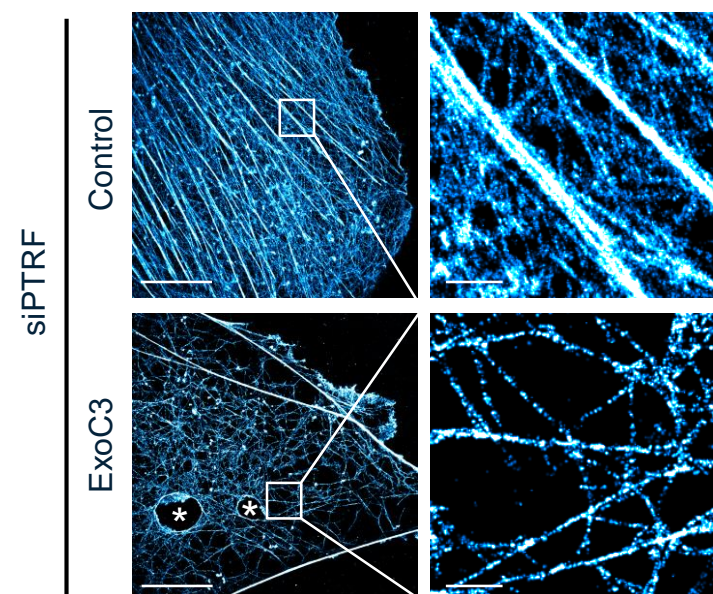
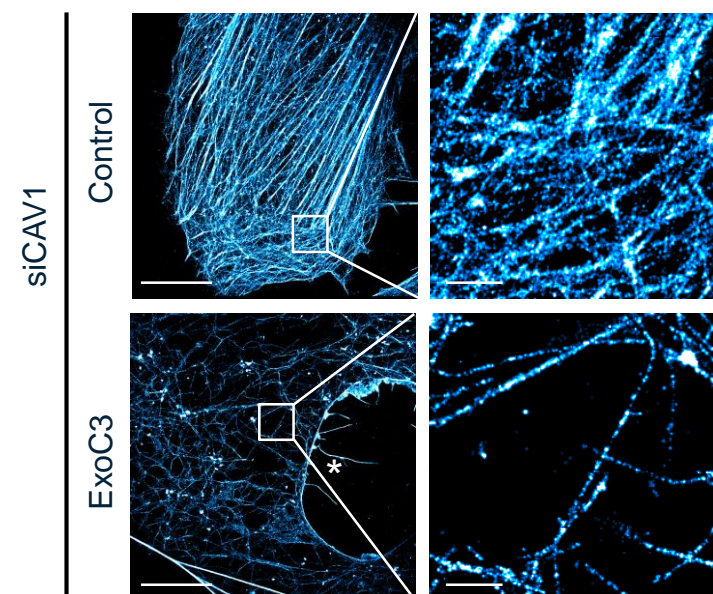
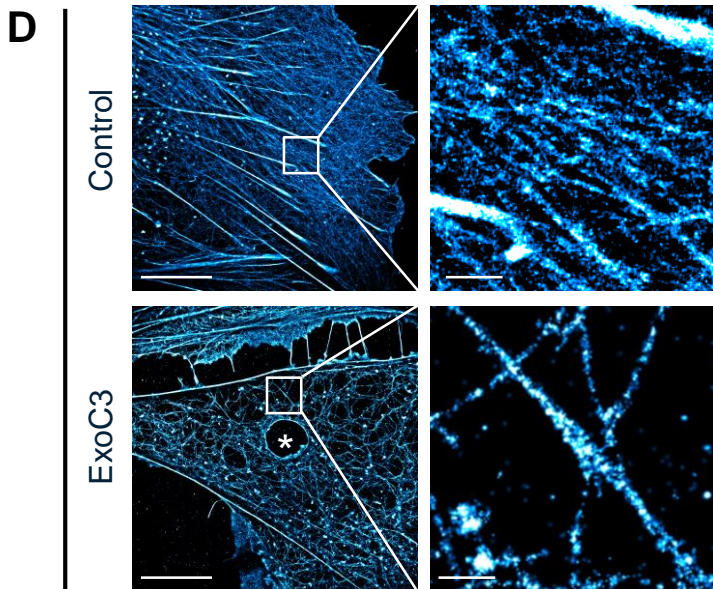
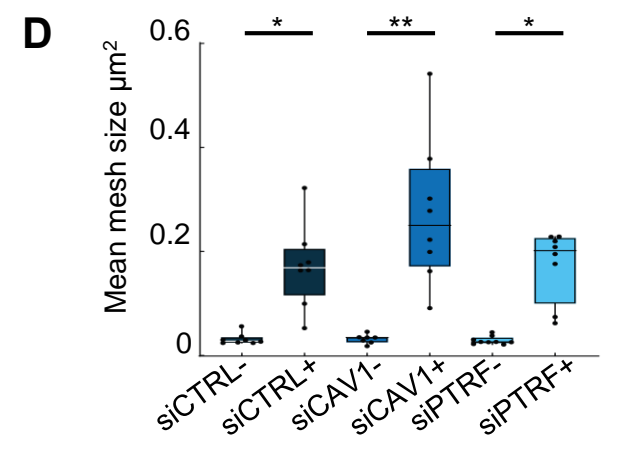
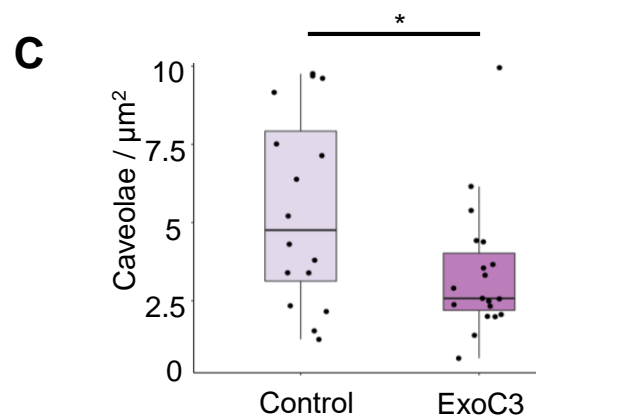
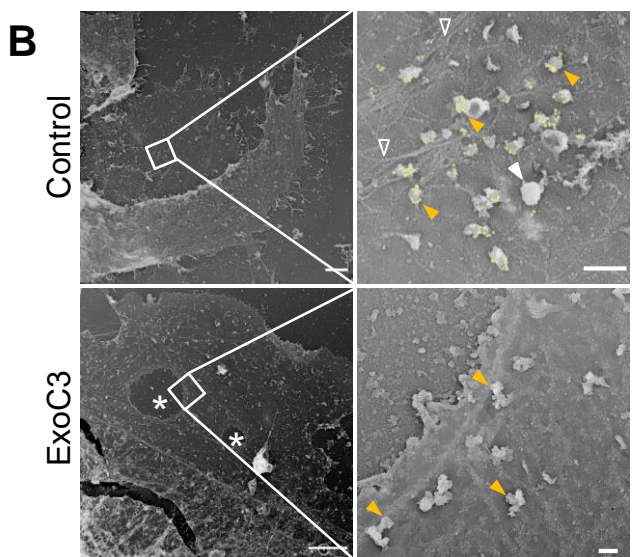
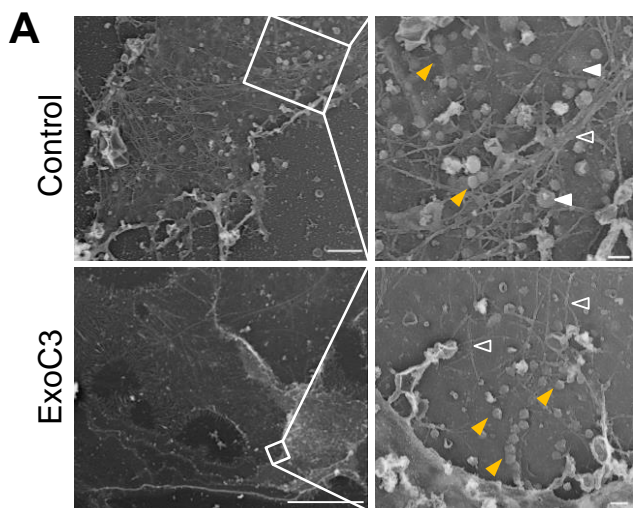
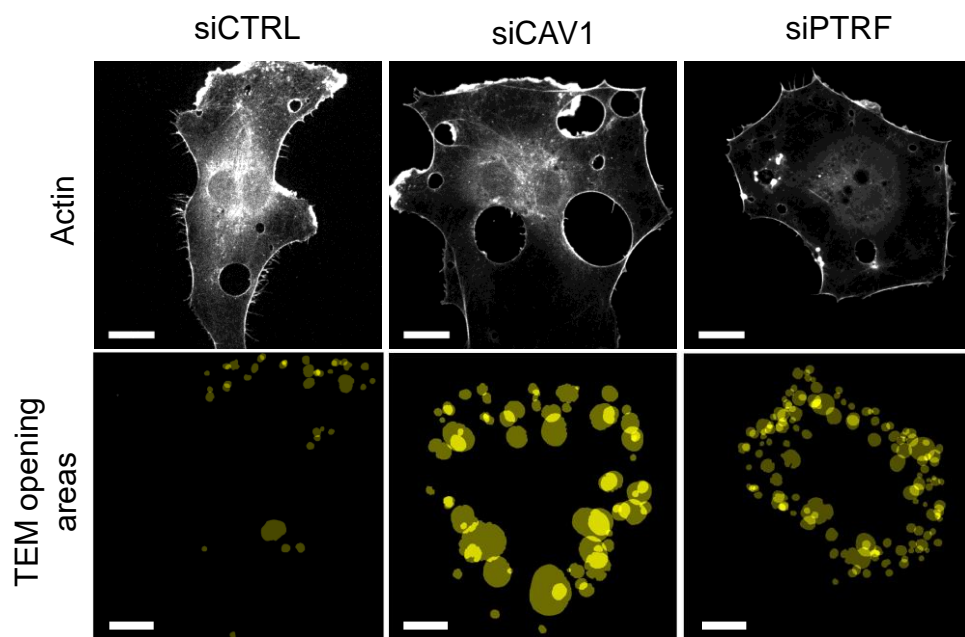


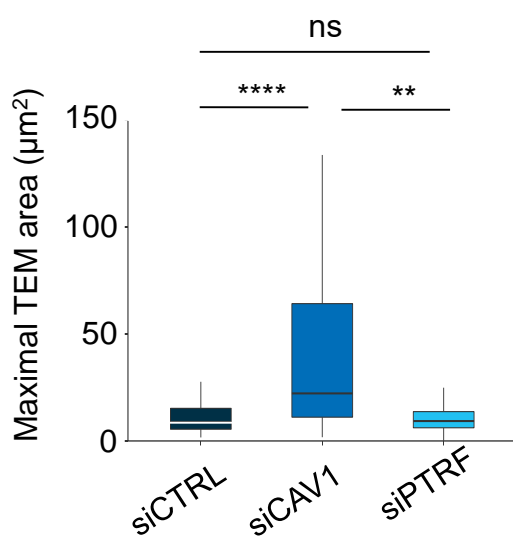
Figure 4



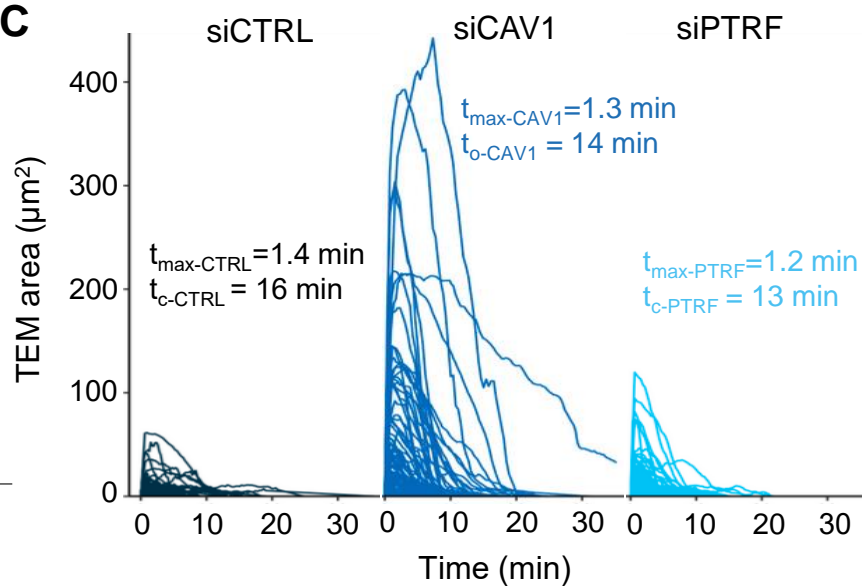
**A**



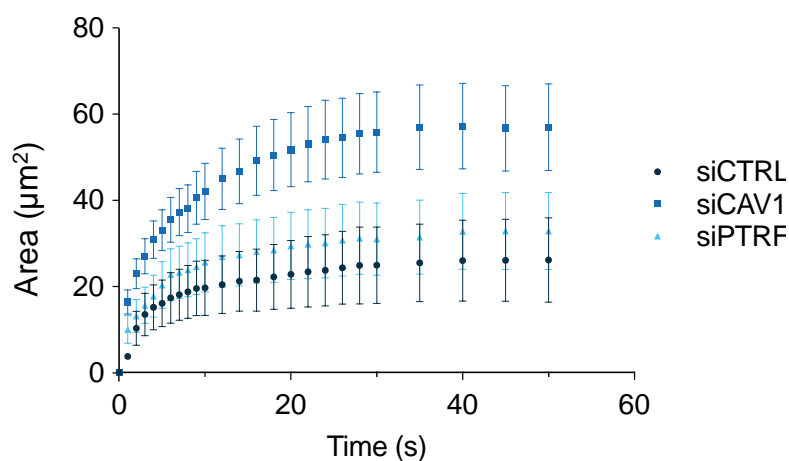
**B**



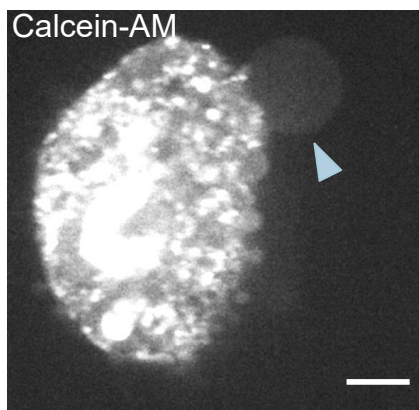
**C**



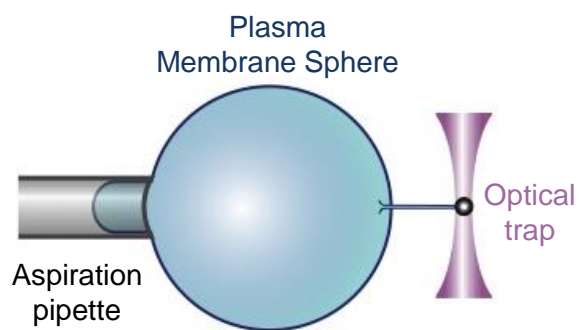
**D**



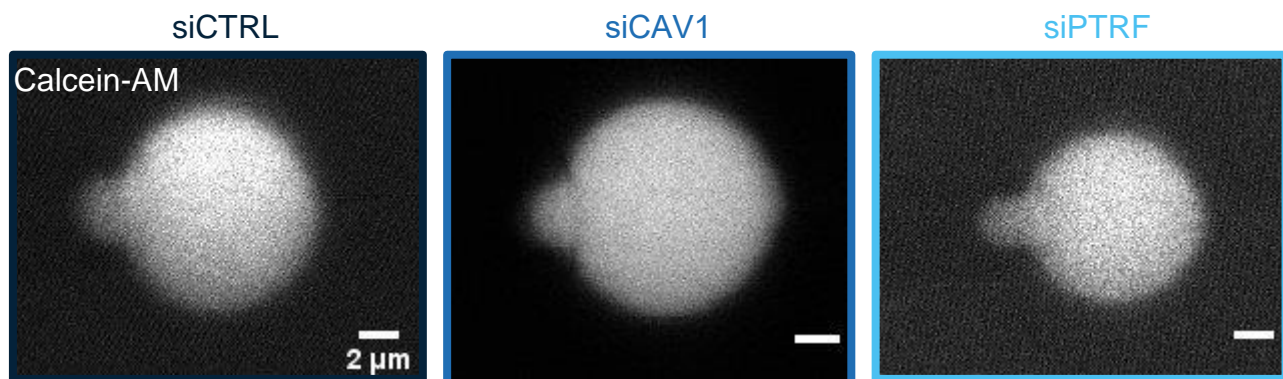
**A**



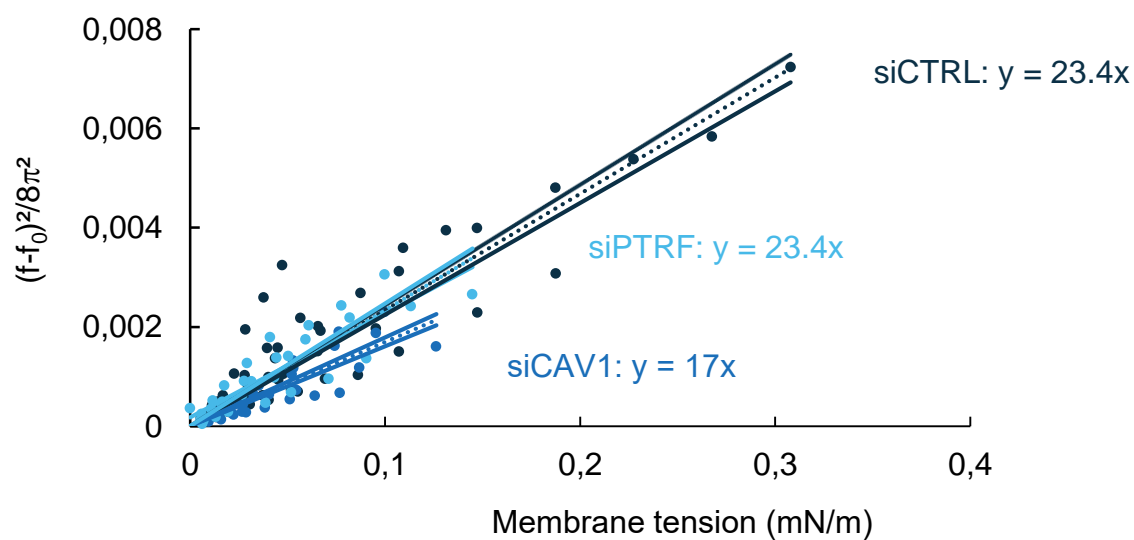
**B**

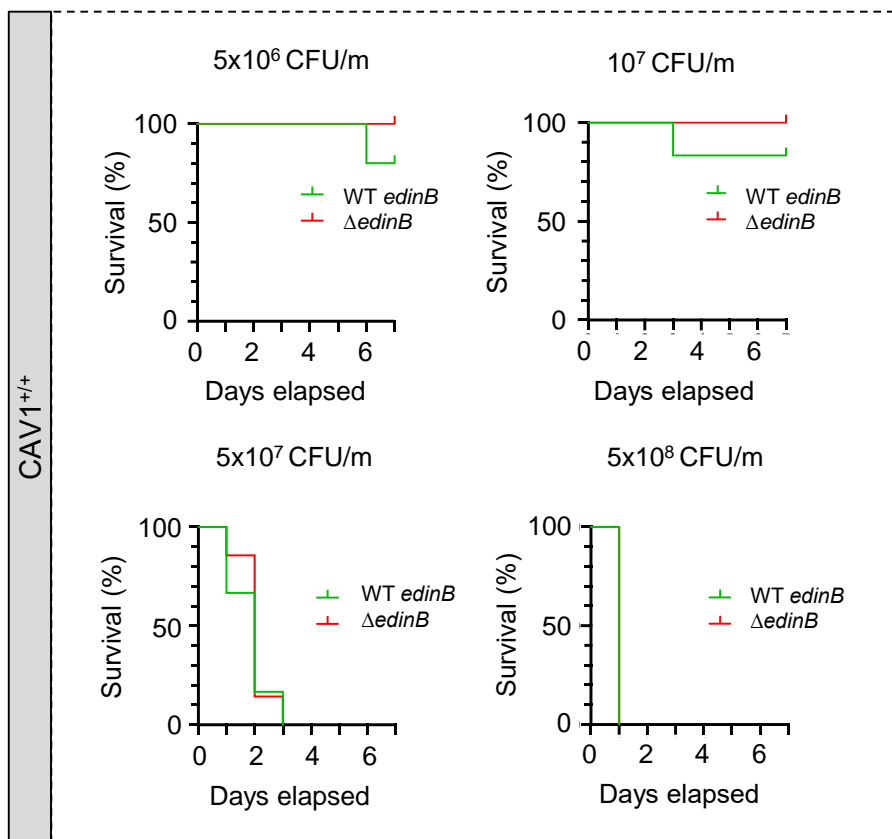


**C**

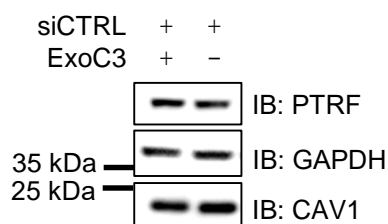


**D**

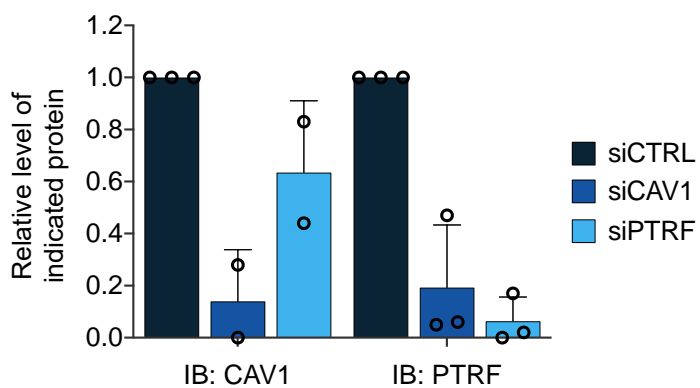
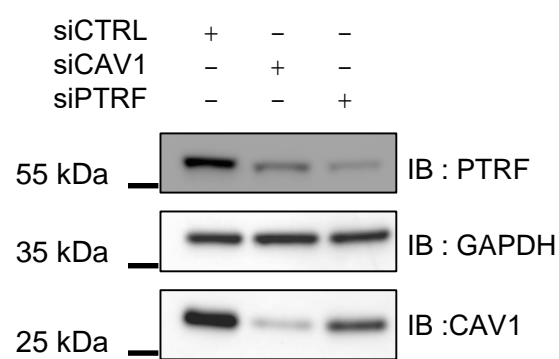




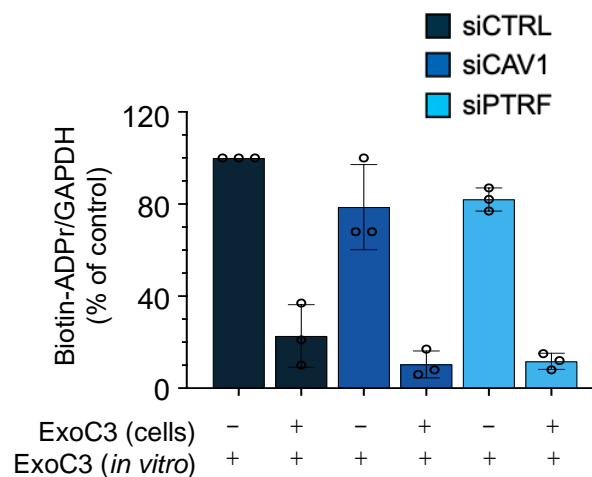
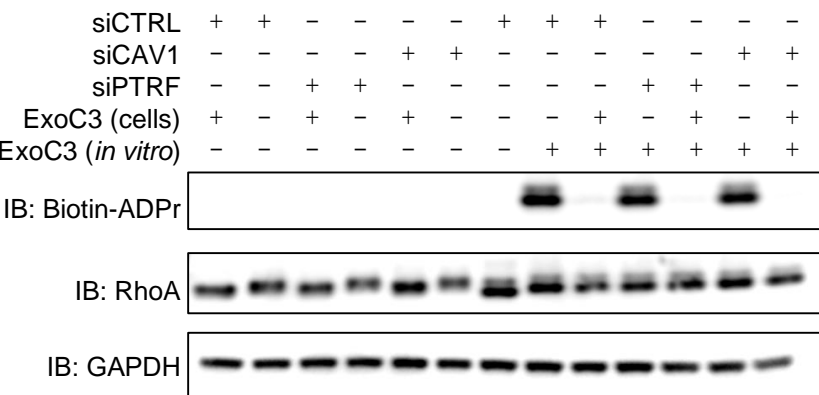
**A**



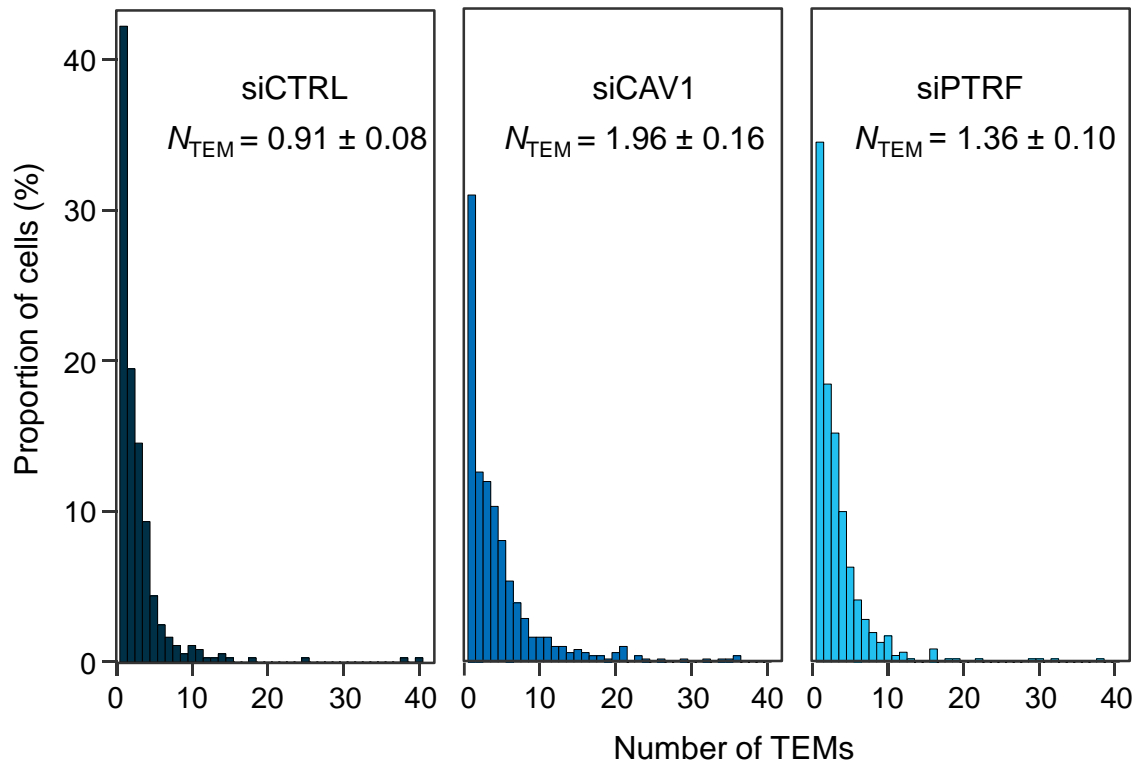
**B**



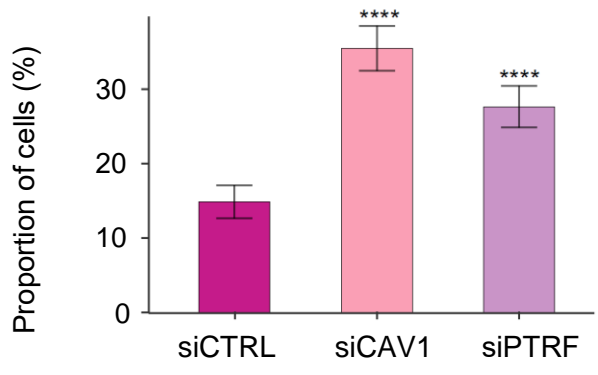
**C**



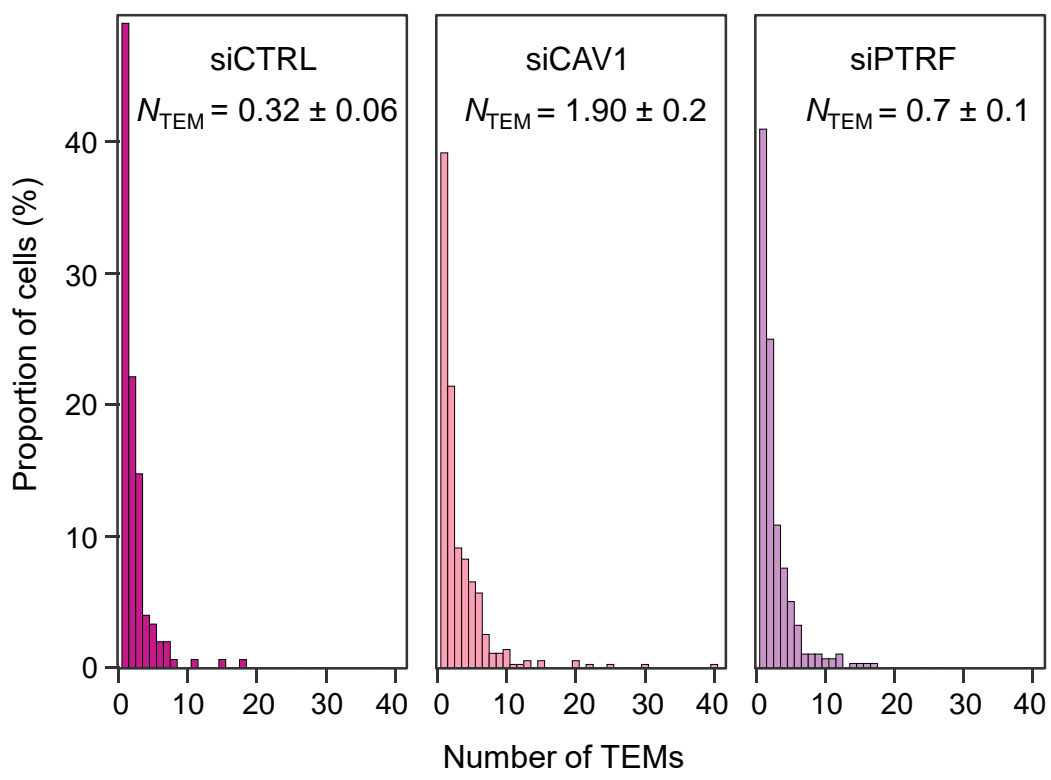
**A** ExoC3 treatment



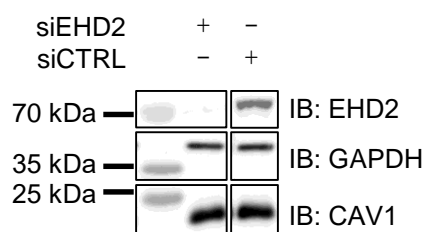
**B** EDIN treatment



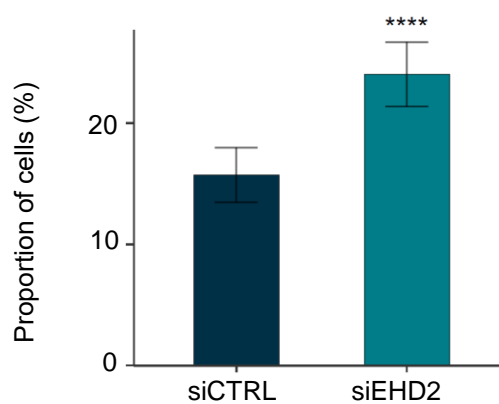
**C** EDIN treatment



**A**



**B** ExoC3 treatment



**C** ExoC3 treatment

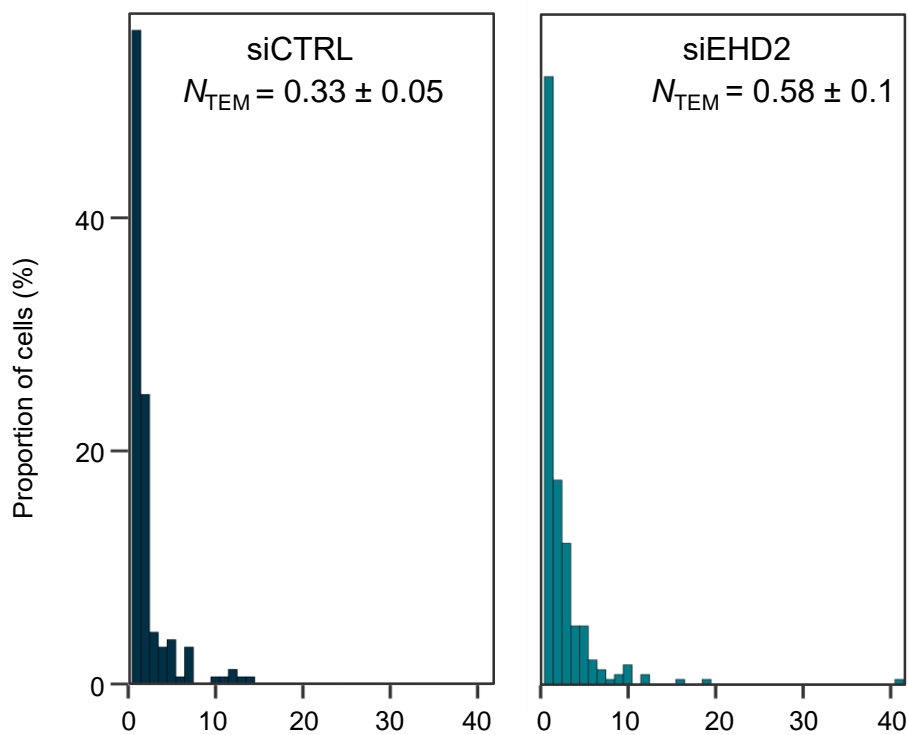


Figure S5

