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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

Authors

Camille Morel¹, Eline Lemerle², Feng-Ching Tsai³, Thomas Obadia^{4,5}, Nishit Srivastava⁶, Maud Marechal¹, Audrey Salles⁷, Marvin Albert⁸, Caroline Stefani⁹, Christophe Lamaze¹⁰, Stéphane Vassilopoulos², Matthieu Piel⁶, Patricia Bassereau³, David Gonzalez-Rodriguez¹¹, Cécile Leduc^{12*}, Emmanuel Lemichez^{1*}

Affiliations

¹ Institut Pasteur, Université Paris Cité, CNRS UMR6047, INSERM U1306, Bacterial Toxins Unit, Department of Microbiology, F-75015 Paris, France.

² Sorbonne Université, INSERM UMR U974, Institut de Myologie, Centre de Recherche en Myologie, F-75013 Paris, France.

³ Institut Curie, PSL Research University, CNRS UMR168, Laboratoire Physico-Chimie Curie, F-75005 Paris, France.

⁴ Institut Pasteur, Université Paris Cité, Bioinformatics and Biostatistics Hub, F-75015 Paris, France.

⁵ Institut Pasteur, Université Paris Cité, G5 Infectious Diseases Epidemiology and Analytics, F-75015, Paris, France.

⁶ Institut Curie and Institut Pierre Gilles de Gennes, PSL Research University, Sorbonne University, CNRS UMR144, F-75005 Paris, France.

⁷ Institut Pasteur, Université de Paris Cité, Unit of Technology and Service Photonic BioImaging (UTechS PBI), C2RT, F-75015 Paris, France.

⁸ Institut Pasteur, Université Paris Cité, Image Analysis Hub, F-75015 Paris, France.

⁹ Benaroya Research Institute at Virginia Mason, Department of Immunology, Seattle, USA.

¹⁰ Institut Curie, PSL Research University, INSERM U1143, CNRS UMR3666, Membrane Mechanics and Dynamics of Intracellular Signaling Laboratory, F-75005 Paris, France.

¹¹ Université de Lorraine, LCP-A2MC, F-57000 Metz, France.

¹² Institut Jacques Monod, Université Paris Cité, CNRS UMR7592, F-75013 Paris, France.

*: corresponding authors: emmanuel.lemichez@pasteur.fr and cecile.leduc@ijm.fr

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 μm (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^{-/-}) and their wild-type littermates (CAV1^{+/+}) 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^{-/-} and CAV1^{+/+} 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^{-/-} mouse, recovered from infection induced with 5×10^6 CFU of bacteria/mouse, and all the CAV1^{-/-} and CAV1^{+/+} mice died 24 hours after intravenous injection of 5×10^8 CFU of bacteria/mouse. In contrast, when mice were challenged with an intermediate dose of 5×10^7 CFU/mouse, we found significant differences in susceptibility between CAV1^{-/-} and CAV1^{+/+} mice ($P < 0.001$, Mantel–Cox test). All the CAV1^{-/-} mice died on day 1 post-challenge, whereas the death of CAV1^{+/+} mice was delayed between 1 and 2 days, with one CAV1^{+/+} 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^{-/-} mouse susceptibility to systemic infection. Despite a broad concentration range
151 for the 5 inocula, we recorded no difference in the susceptibility of CAV1^{+/+} mice to either the *S.*
152 *aureus* WT *edinB* or *edinB*-deleted isogenic strain (Δ *edinB*) (Fig. S1). Nevertheless, a lethal dose
153 50 (LD₅₀) of approximately 2.5×10^7 CFU/mouse was established for both strains of *S. aureus* (Fig.
154 1B). Interestingly, when we performed similar experiments with an inoculum of 2.5×10^7
155 CFU/mouse, we found a significant difference in the survival rates of CAV1^{-/-} mice: $n=11/18$ mice
156 infected with *S. aureus* Δ *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/ μm^2 of membrane (s.d. of 2.1 caveolae/ μm^2), compared with 5.4 caveolae/ μm^2 of
228 membrane (s.d. of 3.1 caveolae/ μm^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 ~ 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 **Different impacts of cavin-1 and caveolin-1 on TEM tunnel formation dynamics**

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

(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 (κ) and membrane tension (σ):

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

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 κ 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 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 α -
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 Δ *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*^{tm1Mls}/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 OD₆₀₀ = 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 µL 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 µg/ml heparin and referred to as complete SFM (SFMc). Cells were cultured
435 on gelatine-coated culture ware at 37 °C with 5% CO₂ for as many as six passages. For siRNA
436 transfection, HUVECs were cultured at a density of 38,000 cells/cm². 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 µg/10⁶ cells) in a 4-mm cuvette
445 (CellProjects). Cells were then electroporated at 300 V and 450 µF with one pulse of a GenePulser

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 μ 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 μ -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 μ 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 T_i 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 μ 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 CO₂ 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 Ca^{2+} and then briefly subjected to a concentration of 0.5 mg/mL poly-L-lysine diluted in Ca^{2+} -free
498 Ringer's buffer (Sigma–Aldrich). Poly-L-lysine was removed by washing with 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% 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 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/cm² 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 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 μg of cell lysate with 2
528 μg of ExoC3 and 10 μM 6-biotin-17-NAD⁺ (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 CaCl_2 , 1.5 mM MgCl_2 , 5 mM HEPES, 2 mg/ml glucose, 150 mM NaCl, 10 μM
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 μm) 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 μm 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 μm , 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 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 MgCl_2 ,
568 pH 6.8) for 10 min at 37 $^\circ\text{C}$. Glutaraldehyde was quenched for 7 min at RT (0.1% 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 μm). 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 μm). 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 σ 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 σ 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 κ 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 σ acting on TEMs and the observed projected surface in
 613 our cells. Thus, the parameter κ 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 σ_0 and
 617 κ .

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 μ 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 σ_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.
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Condition	σ_0 ($\mu\text{N/m}$)	A_{max} (μm^2)	N (average)	$(N \cdot A_{\text{max}})^{-1}$ (μm^{-2})
Control	25 ± 10	8.1 ± 0.5	0.90 ± 0.09	0.137 ± 0.022
siCAV1	50 ± 7	23 ± 4	1.92 ± 0.15	0.023 ± 0.006
siPTRF	29 ± 7	9.3 ± 0.7	1.34 ± 0.11	0.080 ± 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 σ_0 for the control case is obtained from
 651 earlier estimates (65). The increase in σ_0 for siCAV1 and siPTRF cell membranes is deduced from
 652 our experimental data using Eq. 9. The TEM maximum area, A_{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_{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 $P > 0.05$; * : $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: ≤ 0.0001 .

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H2: Supplementary Materials

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Fig. S1. CAV1^{+/+} 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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942 and C.Le. designed the experiments and supervised the project. E. Lemichez, C.Le., D. G-
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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^{-/-} mice and CAV1^{+/+} 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 (Δ *edinB*) is deleted. (A) Comparative analysis of CAV1^{-/-} and CAV1^{+/+} mouse susceptibility to *S. aureus* bacteraemia. Groups of CAV1^{-/-} and CAV1^{+/+} mice were challenged via intravenous injection of 5×10⁶ CFU/mouse ($n=10$ CAV1^{+/+} mice vs. $n=9$ CAV1^{-/-} mice), 5×10⁷ CFU/mouse ($n=7$ CAV1^{+/+} mice vs. $n=9$ CAV1^{-/-} mice) or 5×10⁸ CFU/mouse ($n=7$ CAV1^{+/+} mice vs. $n=8$ CAV1^{-/-} mice). After administering a dose of 5×10⁷ CFU/mouse, we recorded a significant increase in CAV1^{-/-} susceptibility to infection compared with that of the CAV1^{+/+} littermates. Log-rank test (Mantel–Cox), $P < 0.0011$ at 5×10⁷ CFU/mouse (1 experiment). (B) Lethal dose 50 (LD₅₀) of WT *edinB* or Δ *edinB* strains administered to mice. Mice were injected with 2.5×10⁷ CFU/mouse ($n=12$ mice infected with either WT *edinB* or Δ *edinB* strains, 2 experiments). There were no significant difference in the survival kinetics between WT *edinB*- and Δ *edinB*-infected animals, as determined by log-rank test (Mantel–Cox). (C) Comparative analysis of the susceptibility of CAV1^{-/-} mice to bloodstream infection triggered by either the WT or Δ *edinB* strains. Mice were injected with 2.5×10⁷ CFU/mouse ($n=17$ and 18 mice infected with WT and Δ *edinB* strains, from 2 experiments). Log-rank test (Mantel–Cox) showed a significantly higher susceptibility of CAV1^{-/-} mice to WT *edinB* than to Δ *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 μ 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 μ 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.
1014

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 μm ;
1024 high magnification, 200 nm; ExoC3, 5 μm ; high magnification, 200 nm. (C) Boxplot shows the density
1025 of caveolae per μm^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 μm . High magnification images
1033 are shown in the right panels. Scale bar, 1 μm . (E) Boxplot shows the average mesh size per cell
1034 (μm^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. ** $P < 0.01$, * $P < 0.05$
1037

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 μm .
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 μm . (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 μm . (D) the Force required to pull membrane tubes rescaled to $((f-f_0)^2/8\pi^2)$
 1072 (in 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 κ (in 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.).
 1078

1079 **Table 1: Cell spreading area, volume and height.**

Conditions	Mean cell area (μm^2)	s.d. of the cell area (μm^2)	Mean cell volume (μm^3)	s.d. of the cell volume (μm^3)	Cell height (μm)	s.d. of the height (μm)
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

1080

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^{+/+} mice susceptibility to EDIN-B in Staphylococcal septicaemia.

Kaplan–Meier survival curves with data obtained through 7 days from groups of CAV1^{+/+} 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^{+/+} mouse susceptibility to bloodstream infection with either the WT *edinB* or Δ *edinB* strain. Mice were injected with 5×10^6 CFU/m (n=5 mice infected with the WT *edinB* or Δ *edinB* strain; 1 experiment), 10^7 CFU/m (n=17 and n=19 mice infected with the WT *edinB* or Δ *edinB* strain, respectively; 3 experiments), 5×10^7 CFU/m (n=19 or n=18 mice infected with the WT *edinB* or Δ *edinB* strain; 3 experiments), and 5×10^8 CFU/m (n=4 or n=5 mice infected with either the WT or Δ *edinB* strain, respectively; 1 experiment). There were no significant differences in the kinetics of survival between the WT and Δ *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⁺. 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_{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_{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

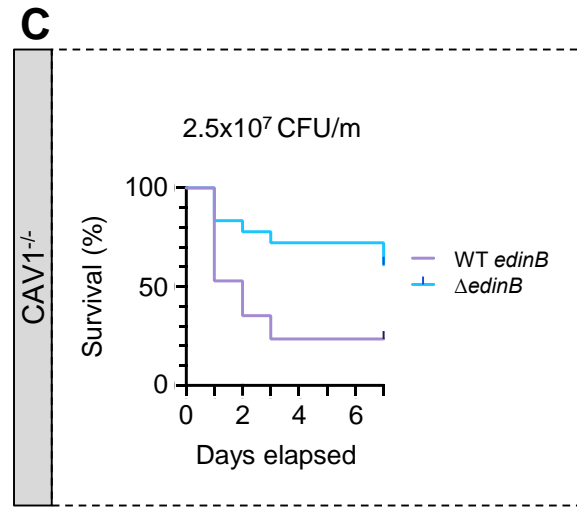
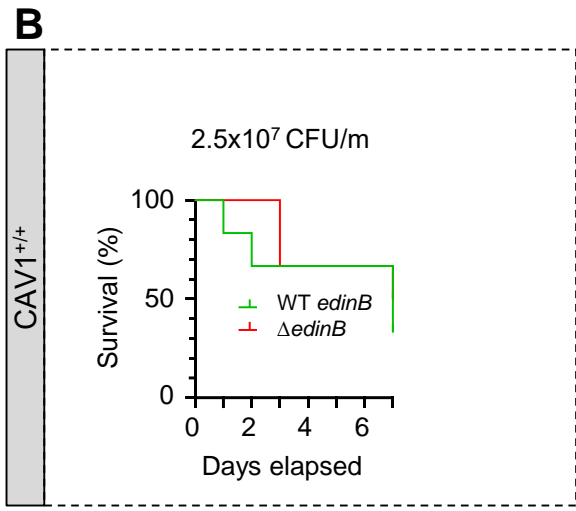
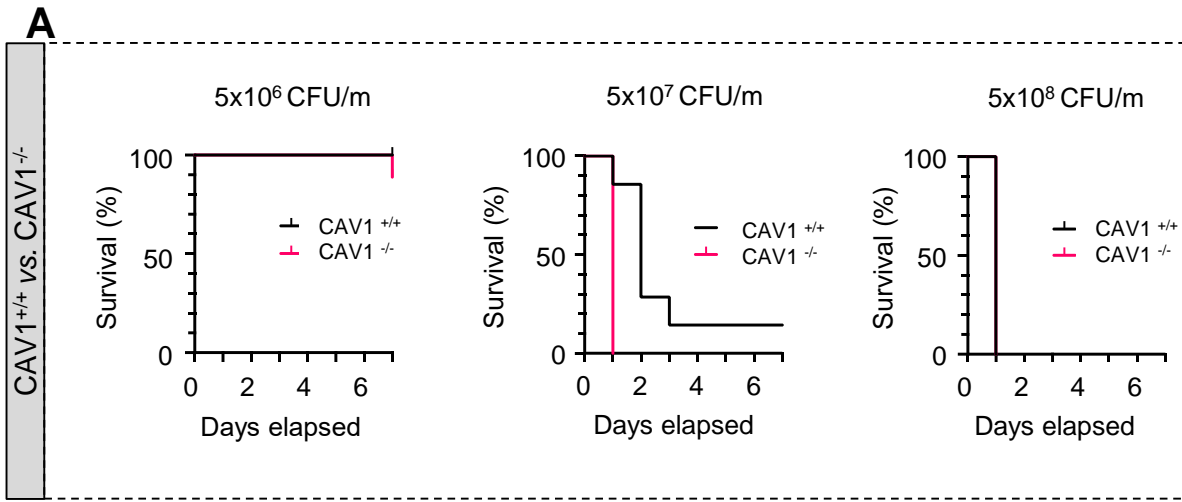
1131 groups. (C) The graph shows the distribution of HUVECs between classes as defined by TEM
1132 density with ranging from a group of cells displaying only 1 TEM and those for which the density
1133 increased by a factor of 1 through those cells with 40 TEMs (y-axis). HUVECs were transfected
1134 with siCTRL, siCAV1 or siPTRF before treatment with ExoC3. The graph includes values the
1135 geometric means (N_{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_{TEM} values, ****P<0.0001 (n >986 cells per
1138 condition from 5 independent experiments).

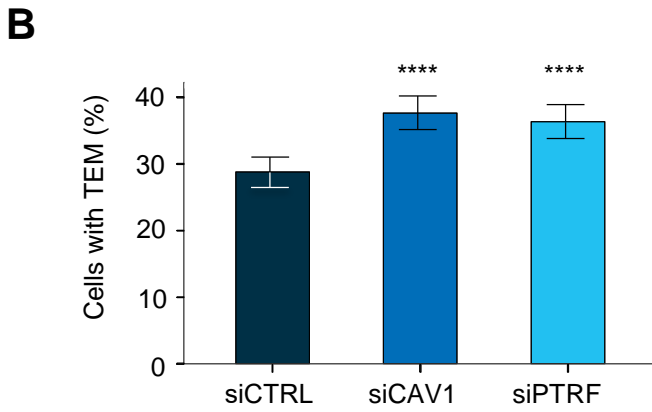
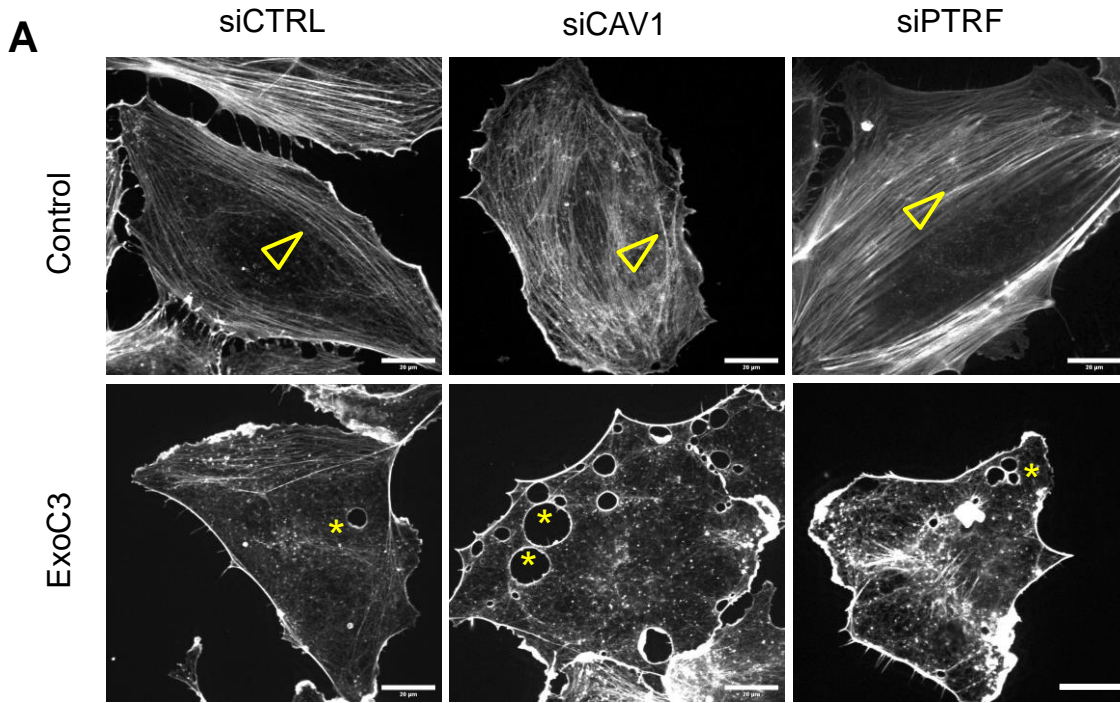
1139
1140 **Fig. S4. Depletion of the caveolar accessory component EHD2 increases TEM formation.**

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_{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_{TEM} values, ****P<0.0001 (n= 1000 cells per condition from 5 independent
1155 experiments).

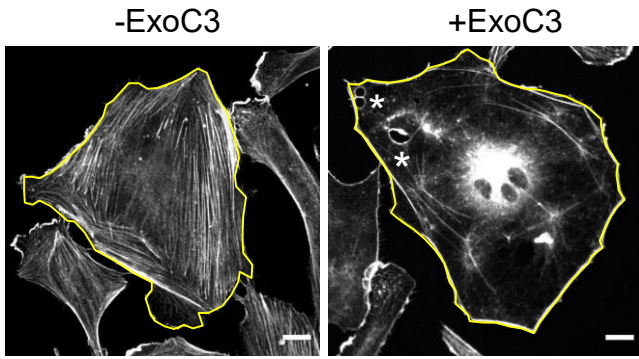
1156
1157 **Fig. S5. Caveolin-1 controls the TEM opening speed and maximum size.**

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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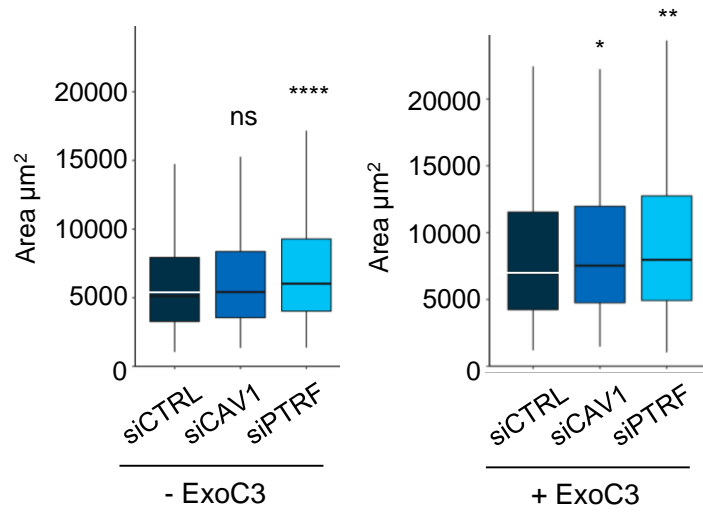




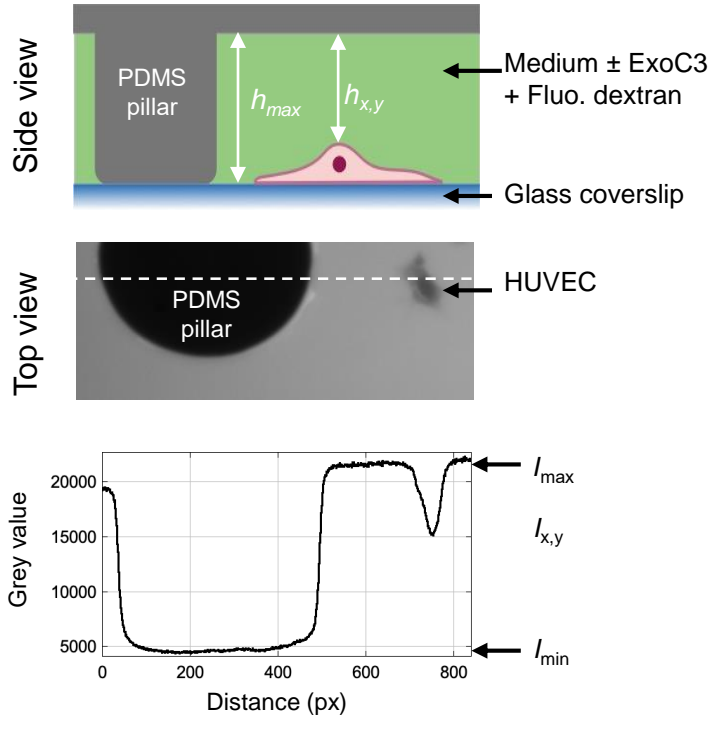
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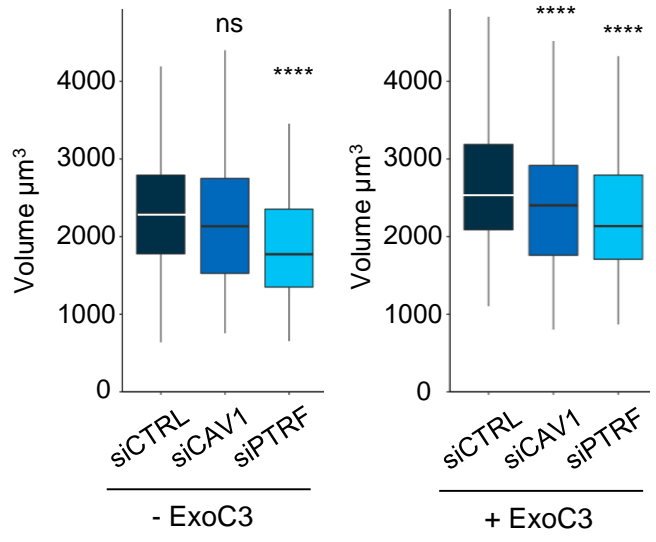
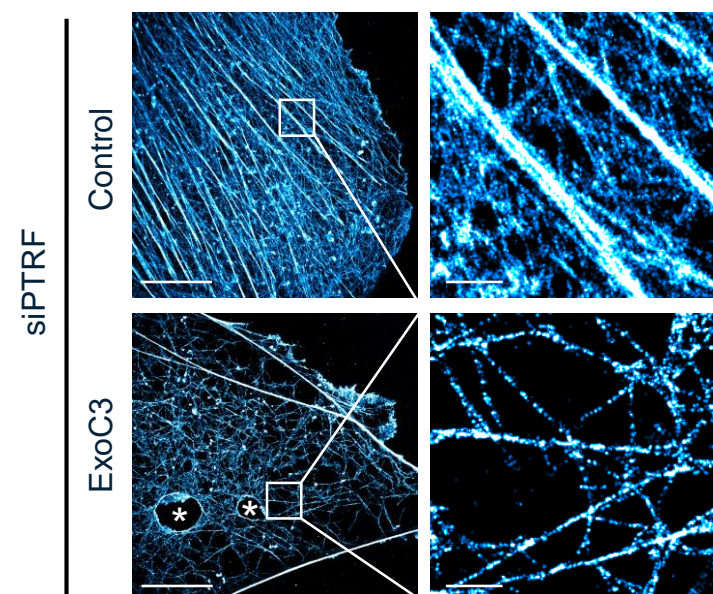
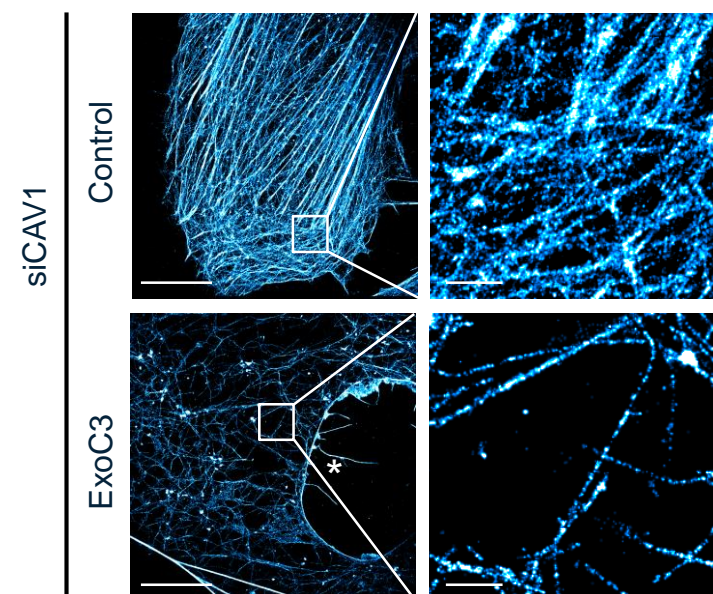
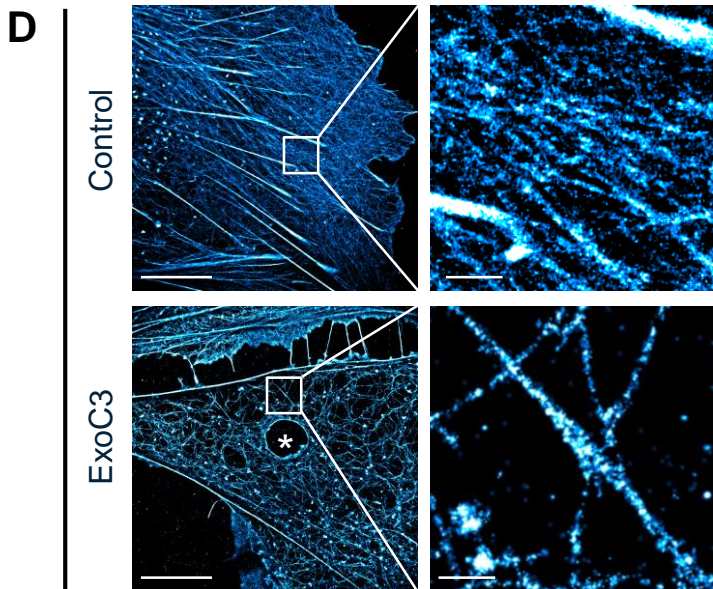
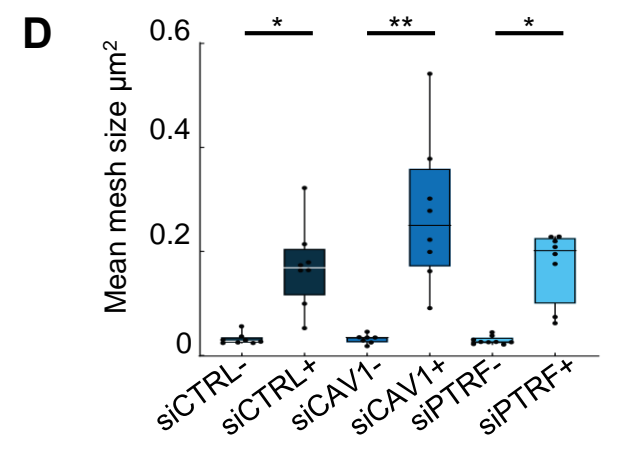
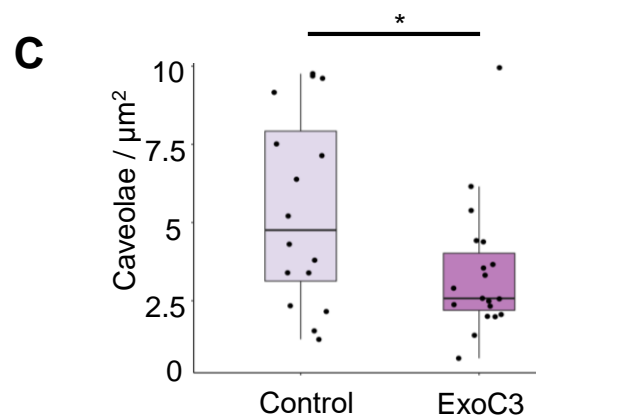
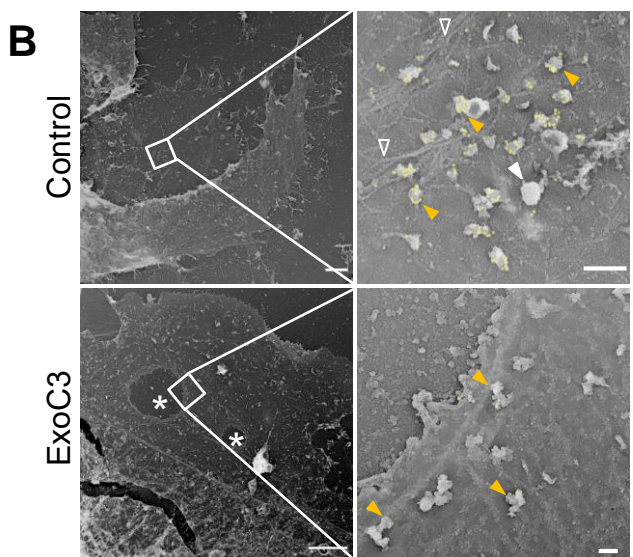
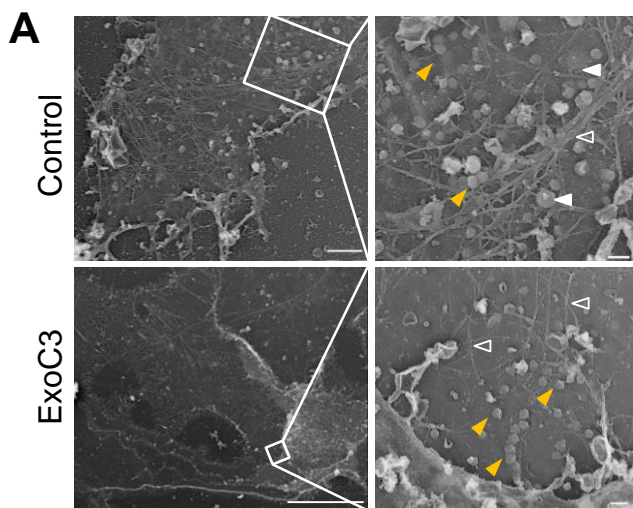
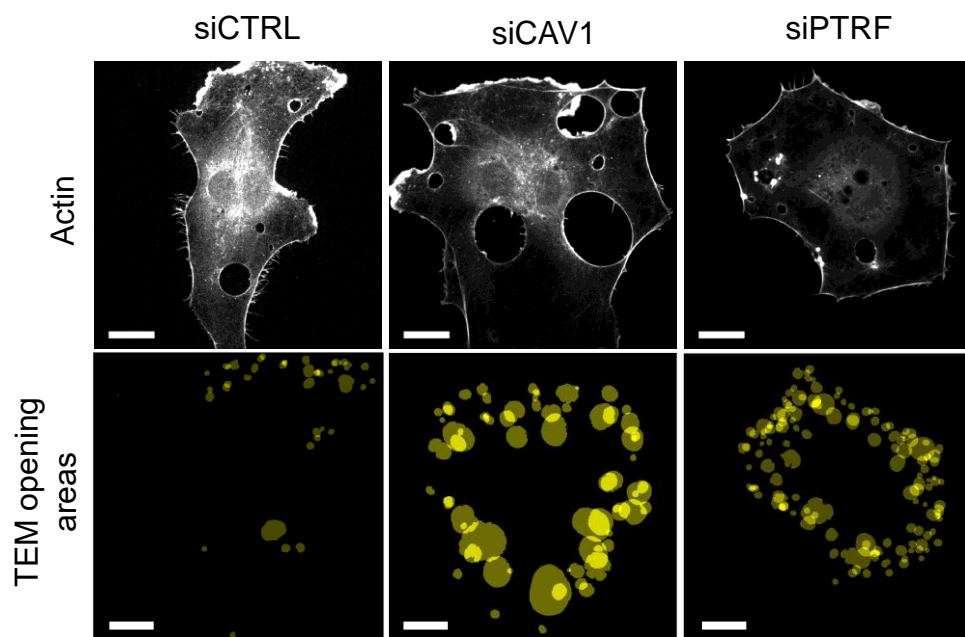


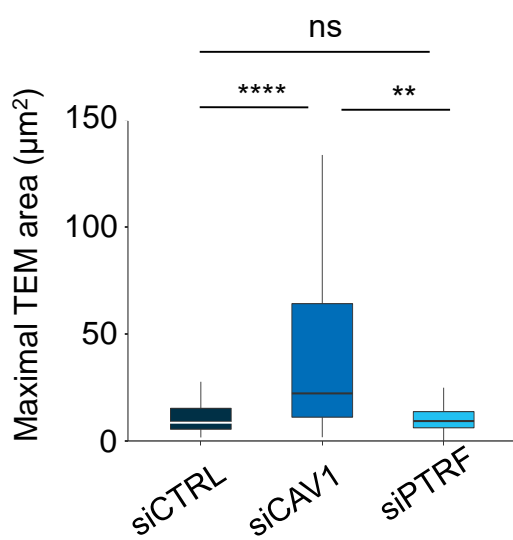
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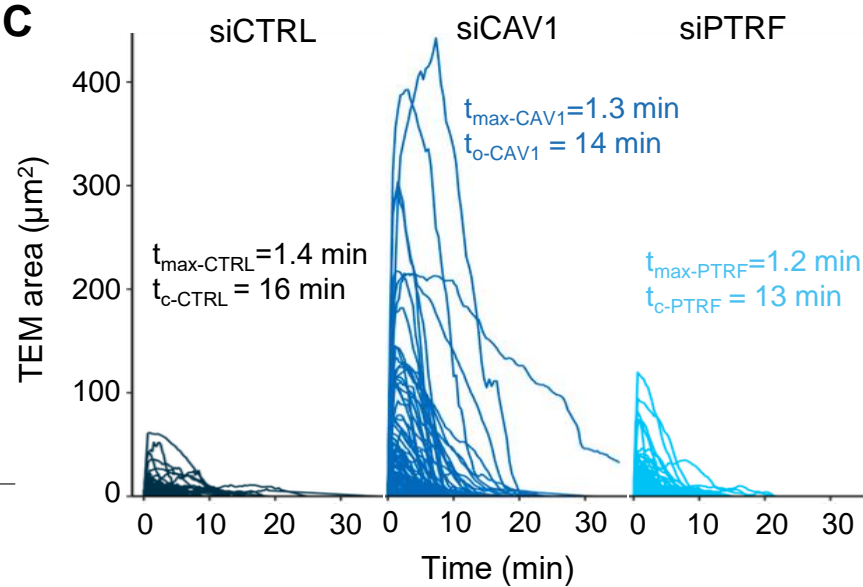
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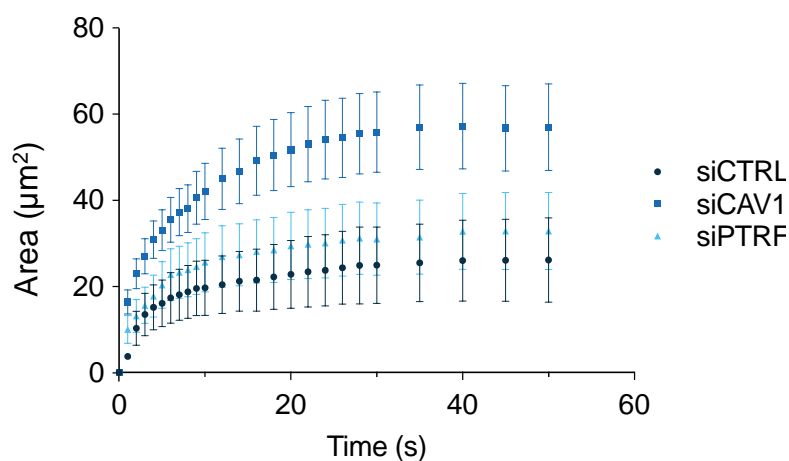
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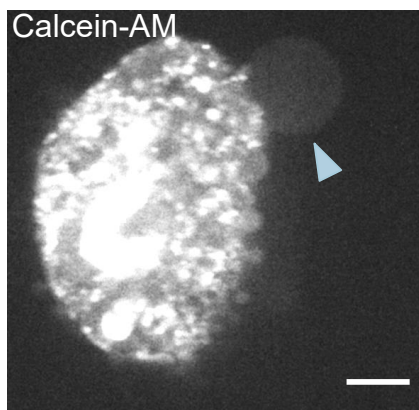
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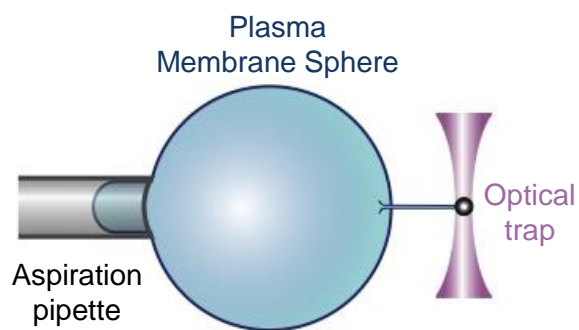
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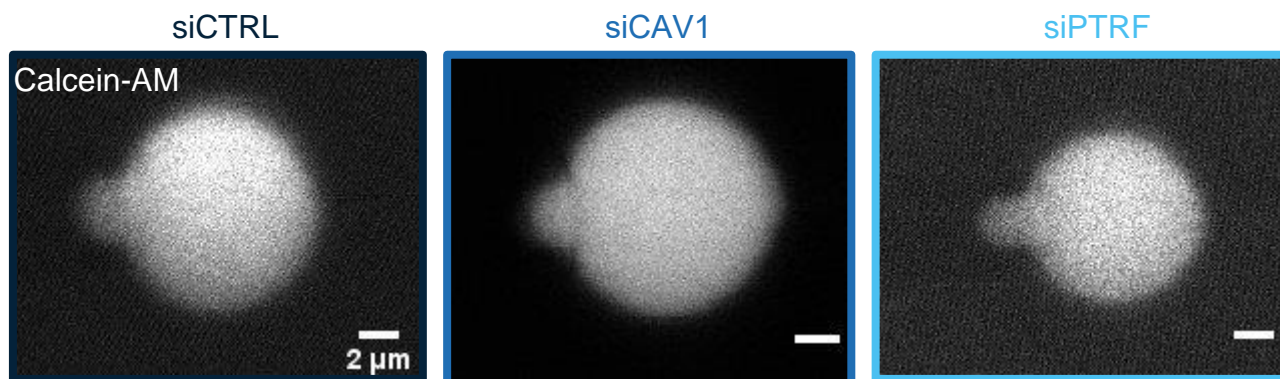
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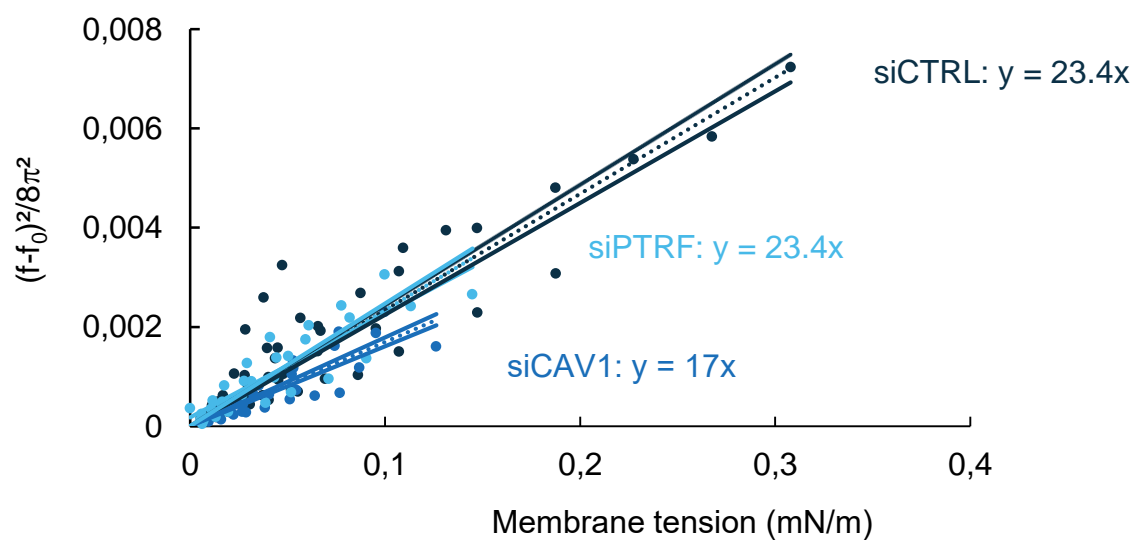
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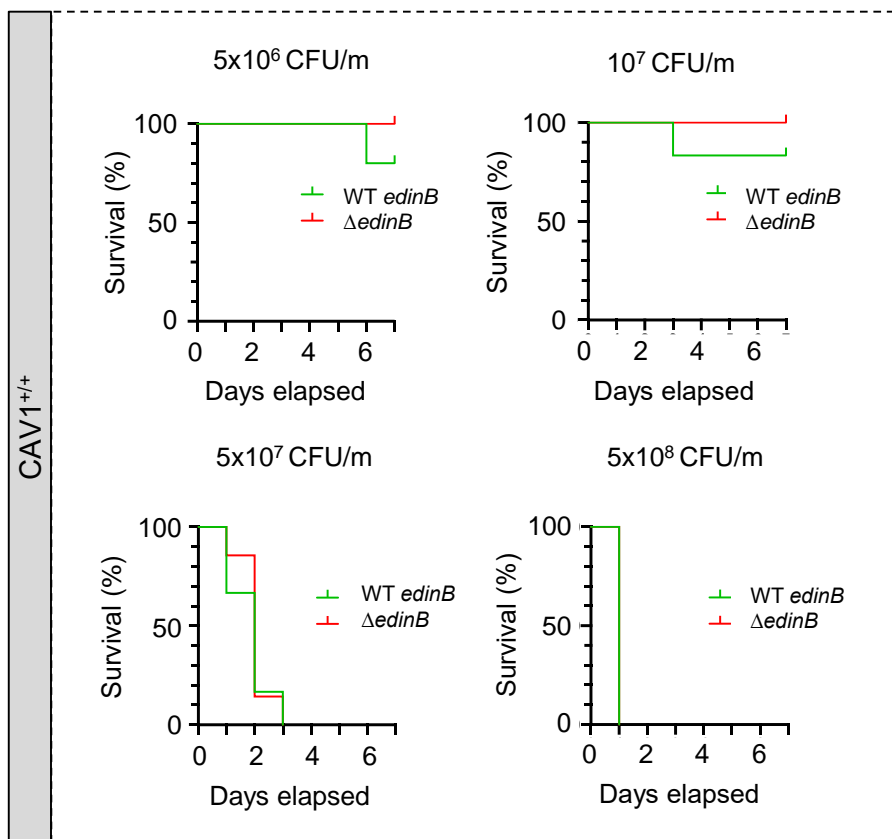


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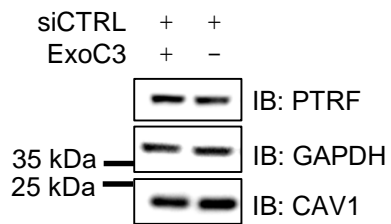


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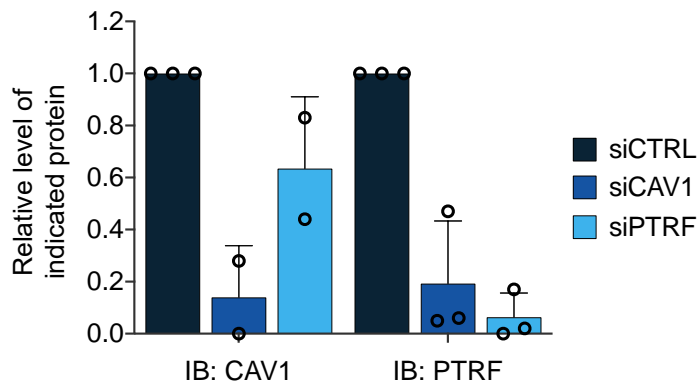
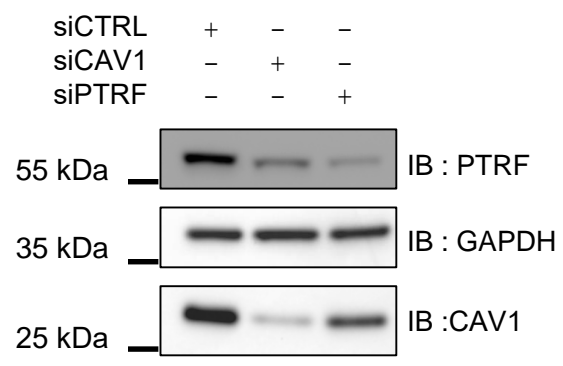




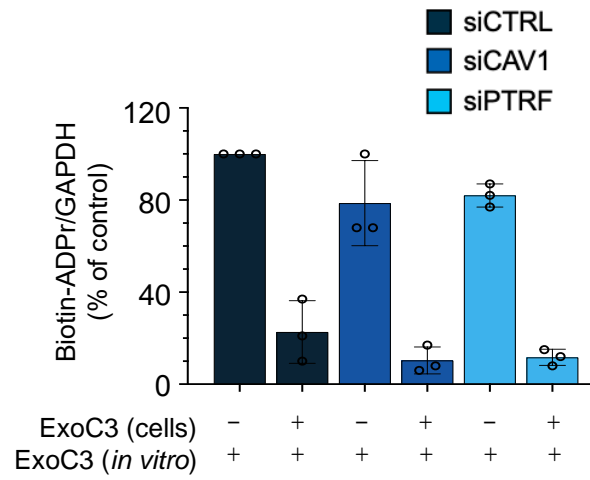
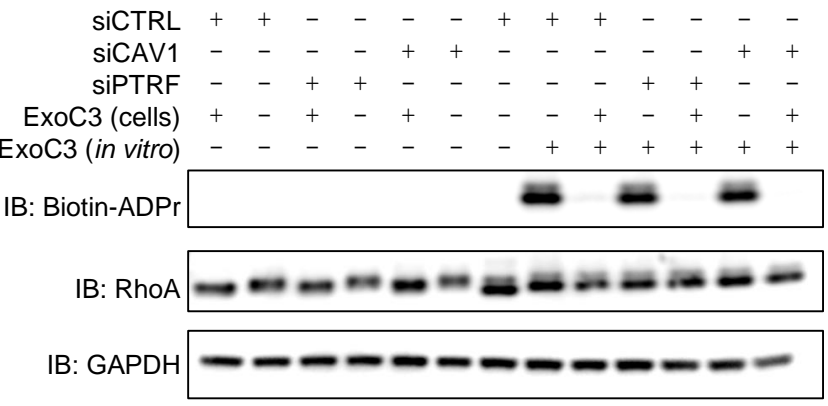
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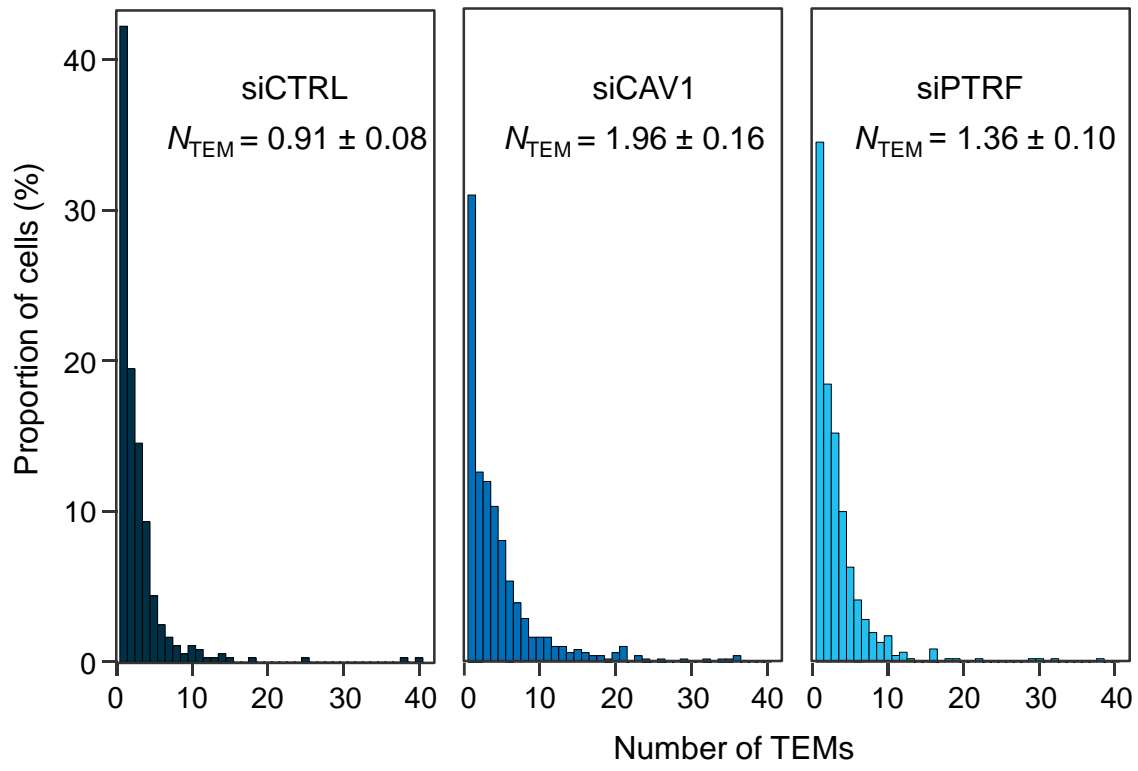
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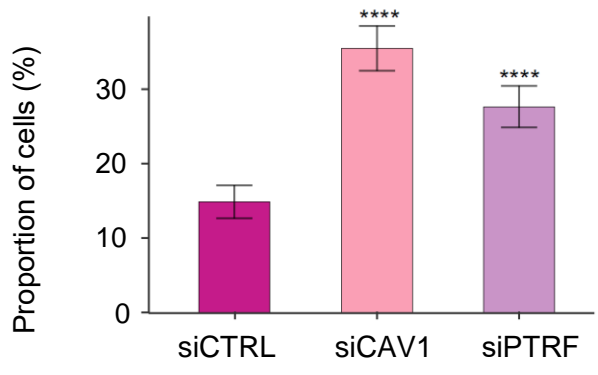
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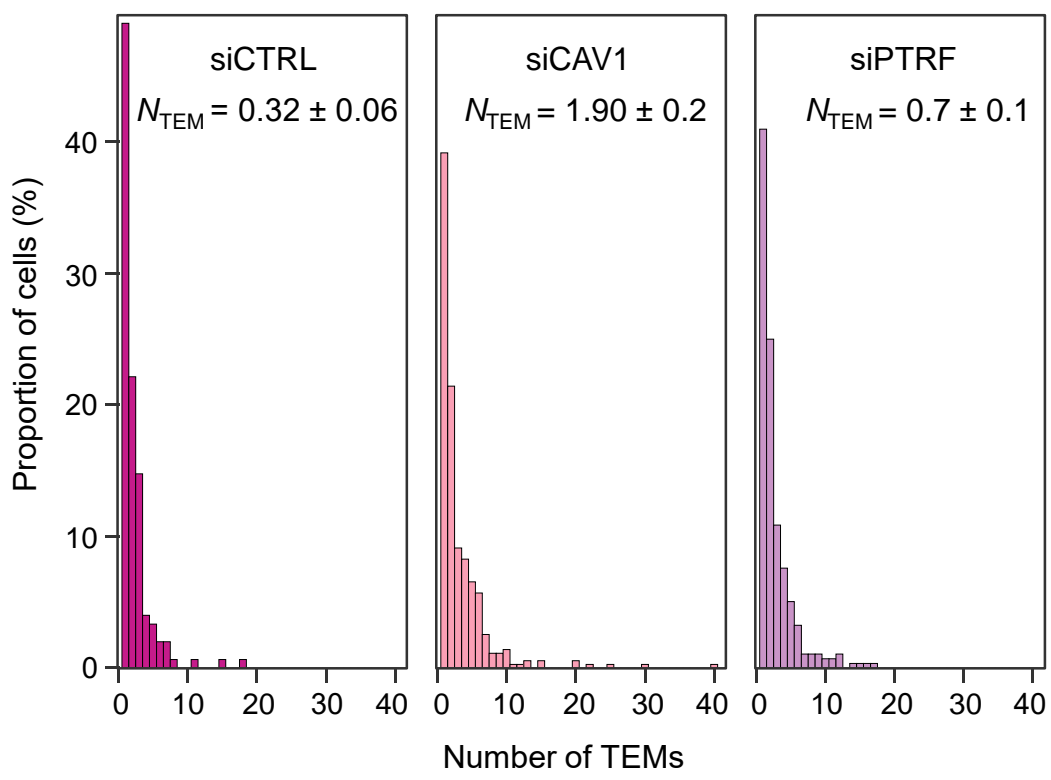
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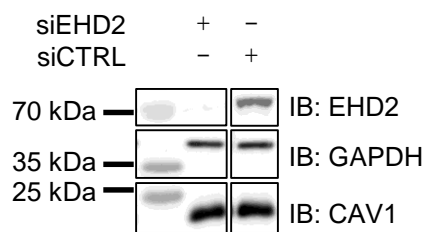
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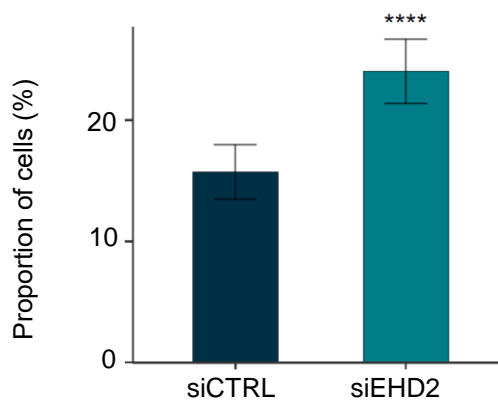
C EDIN treatment



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B ExoC3 treatment



C ExoC3 treatment

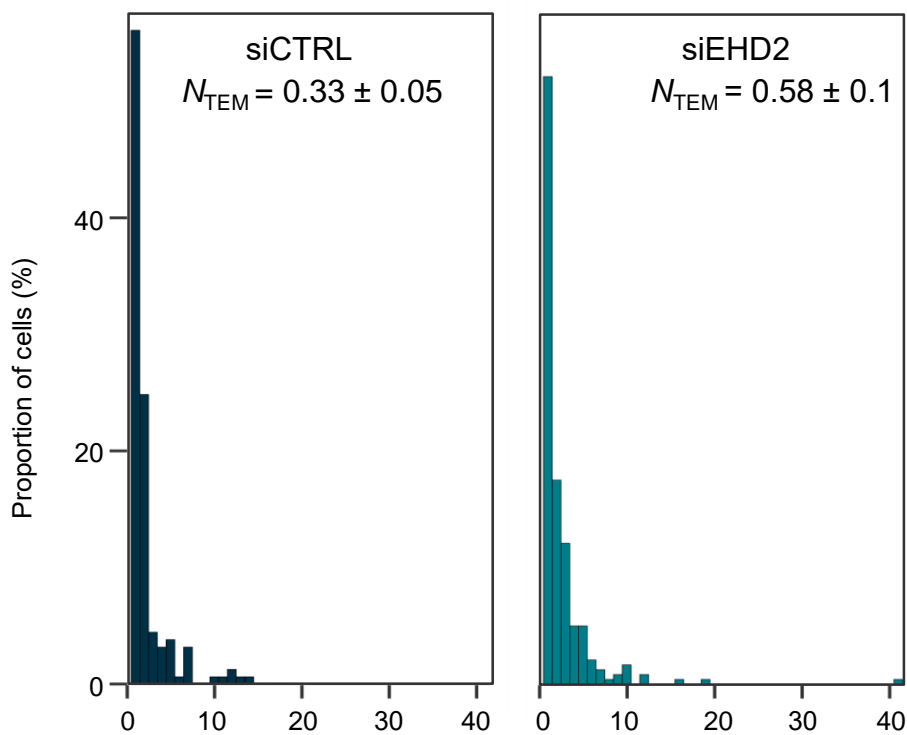


Figure S5

