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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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41 Abstract

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

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57 MAIN TEXT

58 59

60 Introduction

- 61 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 that are 60-80 nm in diameter and large gaps between transendothelial cells that are several 66 micrometres wide (4). Transcellular pores, which contribute to aqueous humour outflow and the 67 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).
- 72

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 μ m (8–10). This group of TEM tunnel-forming toxins includes Clostridium botulinum exoenzyme C3 (ExoC3), which is representative of bacterial ADP 76 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 forces applied to the dorsal region of cells are sufficient to bring apposing membranes into close 82 contact, leading to the opening of TEMs in the presence or absence of EDIN (14). The magnitude 83 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 meshwork trigger cell spreading and a reduction in cell height, which favour the spontaneous 86 formation of TEM tunnels (8, 15). Although TEM tunnels have not yet been visualized in vivo, 87 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.

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Applying concepts discovered via soft matter physics to cellular microbiology enables us to define mechanical constraints and discriminate purely physical processes from those induced by activated cellular factors (*19*, *20*). The phenomenological analogy of TEM tunnel formation and dry patches formed on solid surfaces by viscous liquid dewetting highlights the importance of membrane 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.

105 Caveolae are mechano-regulators of the membrane that spontaneously flatten to accommodate acute mechanical loads and thereby prevent membrane rupturing (22, 23). Caveolin complexes, 106 107 which are essential components in caveolae, insert into the inner leaflet of the lipid bilayer plasma 108 membrane (23, 24). Caveolin-1 homo-oligomers form flat discoid complexes anchored to interfaces 109 (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 110 with caveolin-1 (25–29). Membrane invaginations at caveolae are further stabilized by the assembly 111 112 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 113 mechano-sensing of and adaptation to haemodynamic forces (33, 34). Caveolin-1-deficient mice 114 showed an increase in the eNOS signalling critical for RhoA activation via nitration of 115 116 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-117 118 pathogen interactions (38-40). Considering their critical functions in ensuring membrane mechano-119 relaxation in response to acute mechanical loads (22), caveolae are interesting candidates for the 120 mechano-regulation of TEM tunnels that form upon RhoA inhibition. Together, these findings point 121 to the importance of further characterizing caveolae and the caveolae and caveolin-1 interplay 122 during infection and gaining more information on membrane mechano-regulation. 123

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.

131 **Results**

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

134 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-1-135 deficient mice (CAV1^{-/-}) and their wild-type littermates (CAV1^{+/+}) to the wild-type S. aureus strain 136 LUG1799 (WT edinB). LUG1799 belongs to the European lineage ST80 derived from community-137 138 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 139 increasing concentrations of S. aureus strain WT edinB inoculated through 7 days (Fig. 1A). We 140 did not observe any role for caveolin-1 in mice infected with the predicted nonlethal or lethal 141 inoculum levels. Indeed, all the mice, except one CAV1^{-/-} mouse, recovered from infection induced 142 with 5 $\times 10^6$ CFU of bacteria/mouse, and all the CAV1^{-/-} and CAV1^{+/+} mice died 24 hours after 143 144 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 145 between CAV1^{-/-} and CAV1^{+/+} mice (P < 0.001, Mantel–Cox test). All the CAV^{-/-} mice died on day 146 1 post-challenge, whereas the death of CAV1^{+/+} mice was delayed between 1 and 2 days, with one 147 148 $CAV1^{+/+}$ mouse surviving. The loss of caveolin-1 expression thus sensitized the mice to systemic 149 infection by S. aureus-produced EDIN-B. We then explained a possible role for EDIN-B in

enhancing CAV1^{-/-} mouse susceptibility to systemic infection. Despite a broad concentration range 150 for the 5 *inocula*, we recorded no difference in the susceptibility of $CAV1^{+/+}$ mice to either the S. 151 aureus WT edinB or edinB-deleted isogenic strain ($\Delta edinB$) (Fig. S1). Nevertheless, a lethal dose 152 50 (LD₅₀) of approximately 2.5×10^7 CFU/mouse was established for both strains of S. aureus (Fig. 153 1B). Interestingly, when we performed similar experiments with an inoculum of 2.5×10^7 154 CFU/mouse, we found a significant difference in the survival rates of CAV1^{-/-} mice: n=11/18 mice 155 infected with S. aureus $\triangle edinB$ versus n=4/17 mice infected with S. aureus WT (P = 0.0123, 156 Mantel-Cox test) (Fig. 1C). Our study of caveolae-deficient mice reveals the capacity of the EDIN-157 158 B exoenzyme to trigger lethal effects in mice via septicaemia.

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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 TEM tunnel formation. TEM tunnel formation is triggered by EDIN-like factors and ExoC3 from 162 C. botulinum, which is widely used in cell biology studies (Fig. 2A). We first verified that ExoC3 163 treatment exerted no impact on caveolin-1 and cavin-1 expression in treated HUVECs (Fig. S2A). 164 Importantly, the stoichiometry of caveolin-1 and cavin-1 is highly regulated in endothelial cells 165 166 (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 cavin-1/PTRF (siPRTF) on a cellular pool of caveolin-1 was less pronounced, with an approximate 169 60% reduction in caveolin-1 signal in cells treated with siPTRF. (Fig. S2B). Similar outcomes 170 obtained with siCAV1 and siPTRF indicated the importance of caveolae structures, and different 171 172 outcomes indicated specific roles for caveolin-1. We then verified that siRNA-targeted depletion of caveolin-1 and cavin-1/PTRF did not affect the extent of ADP ribosylation of RhoA (Fig. S2C). 173 174 Ultimately, we verified that TEM tunnels were surrounded by an F-actin bundle after both siCAV1 and siPTRF exposure (Fig. 2A). We then quantified the efficiency of TEM tunnel formation in 175 176 siCAV1- or siPTRF-treated cells (Fig. 2B). HUVECs were treated with siCTRL, siCAV1 or 177 siPTRF 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 siPTRF-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_{TEM siCAV1} = 1.96) and siPTRF (N_{TEM siPTRF} = 1.36) groups, compared to that of the 182 siCTRL ($N_{siCTRL} = 0.91$) 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 further determine the importance of caveolae in counteracting TEM tunnel formation, we depleted 184 the caveolae-stabilizing protein EHD2 in cells. The depletion of EDH2 in siEHD2-treated cells did 185 186 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.

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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 membranes, creating TEM nucleation sites (14, 15). The height of cells is proportional to the 193 194 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 treated with ExoC3, we recorded an increase in the spreading area of cells, and this effect was 201 associated with a reduction in the volume of the cells (Fig. 3C-D). Thus, ExoC3 treatment induced 202 203 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 promoted TEM formation to a similar extent. There was a noticeably stronger regulatory effect of 206 207 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 concomitant decrease in cell volume and an increase in cell spreading. 210

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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 a platinum replica via transmission electron microscopy (43). In both naïve and ExoC3-treated 217 218 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. 4A). However, the density of the cytoskeletal filaments was less pronounced in the ExoC3-treated 220 221 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 of caveolae in control and ExoC3-treated cells. In total, we analysed an area $A_{area} > 175 \ \mu m^2$ under 224 225 each condition ($n \ge 16$ membrane areas of independent cells per condition). We found a 1.6-fold decrease in the mean density of caveolae in the ExoC3-treated cells, which exhibited 3.4 226 caveolae/ μ m² of membrane (s.d. of 2.1 caveolae/ μ m²), compared with 5.4 caveolae/ μ m² of 227 228 membrane (s.d. of 3.1 caveolae/µm²) 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 Factin meshwork that formed between well-defined thick actin cables in untreated HUVECs. In 232 contrast, after siRNA treatment, the cells treated with ExoC3 showed profound reorganization of 233 234 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 caveolae invaginations at the membrane and a loosened actin filament mesh. 237

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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 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 246 247 tunnels and postprocessing of variations in TEM tunnel width as a function of time (15). After we performed this analysis, we projected the surfaces of all the TEM tunnels that had formed over a 1-248 249 hour period of recording when they first opened onto the image showing the Lifeact-GFP signal 250 (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 251 wider TEMs in siCAV1-treated cells than in the siPTRF- and siCTRL- treated cells (Fig. 5A). A 252 253 quantitative analysis established a 5.4-fold increase in the maximal TEM formation area under 254 caveolin-1-depleted conditions compared to that under siPTRF and siCTRL conditions (Fig. 5B). 255 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 256 257 1.3 min) and t_{max} siPTRF=1.2 min (s.d. of 1.4 min.). In addition, overall similarities in TEM tunnel 258 formation cycles were observed between 13 and 16 min (Fig. 5C). Altogether, these findings point 259 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 260 261 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 262 resolution to resolve the TEM opening speed (S₀). In good agreement with our aforementioned data, 263 we recorded no significant variation in the TEM opening speed between the siCTRL- and siPTRF-264 treated groups (Fig. 5D). In contrast, we recorded a 2-fold higher TEM opening speed in caveolin-265 266 1-depleted cells $S_{0_{siCAV1}}=2.4 \ \mu m^2/s$ versus $S_{0_{siCTRL}}=1.1 \ \mu m^2/s$ (Fig. 5D). Thus, caveolin-1 specifically controls the TEM opening speed and maximal width. 267

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269 We next inferred changes in membrane mechanical properties due to the depletion of caveolin-1 or 270 cavin-1 using a refined theoretical model of cellular dewetting. This model accounts for the 271 presence of several TEMs opening simultaneously (see the physical model description in the 272 Materials and Methods). Importantly, the model is based on the hypothesis that plasma membrane 273 deformation enabling TEM nucleation and tunnel growth is a function of membrane tension and 274 bending rigidity, within the limit of an absence of contribution of membrane adhesion to cortical 275 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 276 277 meshwork in ExoC3-treated cells (Fig. 4D). As explained in the Materials and Methods section in 278 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 279 280 predicted a significant 2-fold increase in membrane tension in cells treated with siCAV1, and no 281 effect in siPTRF-treated cells. The model also predicted a maximum TEM size that greatly 282 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 283 284 provided an estimate of the variation in bending rigidity. Using values obtained from the siCTRLtreated cells, which were a $\sigma_0 = 2.5 \ 10^{-5} \text{ N/m}$ for the initial membrane tension (45) and $\kappa = 23.4$ 285 k_BT for the bending rigidity (direct measurement, see below), the model predicts a 15% reduction 286 287 in membrane bending rigidity upon silencing of cavin-1 expression; and a 55% reduction after 288 caveolin-1 depletion (Fig. 5D). In conclusion, the physical model points to the dominant function 289 of caveolin-1 in controlling membrane tension and bending rigidity, which stands in contrast with 290 the marginal or negligible effect predicted for cavin-1.

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

Our study of TEM dynamics suggests that caveolin-1 controls membrane mechanical parameters, particularly bending rigidity. Therefore, this parameter was further investigated using a quantitative method based on pulling membrane tethers from cell-derived plasma membrane spheres (PMSs) (22, 46, 47) (Fig. 6A). The use of PMSs allowed us to study the mechanical properties of the plasma membrane without the F-actin cortex (47). PMSs prepared from untreated siCTRL-, siCAV1treated and siPTRF-treated HUVECs were aspirated into a micropipette, allowing to control the membrane tension by changing the aspiration pressure. Membrane tethers were pulled from PMSs using optical tweezers which can be used to measure the force, f, exerted on the membrane tether 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):

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

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

312 **Discussion**

We reported the protective function of caveolae against the lethal effect of EDIN-B obtained 313 from S. aureus in mice with septicaemia. Moreover, the cellular depletion of caveolin-1 and cavin-314 315 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 included a decrease of cell volume and an increase of spreading area. Together these effects 321 decreased the height of the cells and likely favoured the contact of plasma membranes, creating 322 TEM nucleation sites. In summary, this work establishes new distinct functions for caveolin-1 and 323 324 cavin-1-structured caveoale in the regulation of membrane rigidity and cell shape, respectively.

Considering the analysis of variations in the width of the TEMs using the cellular dewetting 325 model, we provided compelling evidence that caveolin-1 controls not only the parameters related 326 327 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 simplifications used in the theoretical model, notably the simplified treatment of physical 332 333 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 conical Ca^{2+} -ATPase SERCA (50). Previously performed simulations also confirmed the softening 338 339 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 (>>10%), 341 which was generally not achievable with the reconstituted membranes described in the 342 aforementioned references.

Caveolin-1 may contribute to plasma membrane rigidification through numerous effects, i.e., by structuring ordered lipid domains or anchoring the membrane to cortical cytoskeletal components. Considering this last point, disruption of the interactions between the plasma membrane and the cell cortex after caveolin-1 depletion is expected to exert little effect upon RhoA 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 decrease in Young's modulus of the cell cortex in ExoC3-treated cells (14), pointing to dominant 350 351 roles for membrane intrinsic mechanical parameters when RhoA is inactivated. Moreover, previous studies have established that caveolae in the membrane are enriched with cholesterol, 352 353 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 membrane through the incorporation of poly-unsaturated acyl chains in phospholipids failed, likely 358 359 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.

Our data point towards a major effect of cavin-1 in regulating the spreading area and the 362 volume of cells, which might be linked to cavin-1 driving caveolae invaginations. Our data showed 363 that ExoC3 treatment induced an increase in cell volume, and cell spreading was enhanced. With 364 siCAV1-treated cells, similar effects were observed but only after ExoC3 treatment. The 365 366 uncoupling of cell spreading and volume decrease could be due to the known permissive effect of RhoA signalling; this swelling could induce the establishment of an outwardly rectifying anion 367 current mediated via the volume-rectifying anion channels (55, 56). Interestingly, a decrease in cell 368 369 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 awaits determination. Our findings suggest that depletion of caveolae, as well as the inactivation of 371 372 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 between the lethal effect of EDIN-B in caveolin-1-deficient animals and the protective function of 378 379 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 permeability, increasing transcellular permeability, and that ADP-ribosyltransferases targeting 382 383 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 leukocydin, is positively associated with the haemoptysis aetiology, which manifests as blood in sputum (61). In line with this finding, a previous study 387 388 suggested that EDIN-B might promote the translocation of S. aureus from lung tissues to the bloodstream (18). Recently, a human genetics study pinpointed the impact of haploinsufficiency of 389 390 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. Collectively, our findings ascribe caveolin-1 expression level variations to key functions in *S. aureus* infection.

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402 Materials and Methods

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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

Adult male and female B6.Cg-Cav1tm1Mls/J mice (strain #: 007083, Jackson Laboratories) and 413 414 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 $OD_{600} = 1$ 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.

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

430431 Cell culture, transfection and toxicity

Primary human umbilical vein endothelial cells (HUVECs, PromoCell) were cultured in human 432 433 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 transfection, HUVECs were cultured at a density of 38,000 cells/cm². ON-TARGETplus smart pool 436 siRNA (Dharmacon) targeting human caveolin-1 (L-003467-00-0005), EHD2 (L-016660-00-437 0005), cavin-1/PTRF (L-012807-02-0005) or control RNAi SR-CL000 (Eurogentec) was used at 438 439 100 nM via magnetofection technology (OZ Biosciences) following the manufacturer's instructions 440 in serum-free OptiMEM (Gibco). When necessary, cells were electroporated 24 h postmagnetofection and then used from 48 to 54 h post-magnetofection. HUVEC electroporation of 441 plasmids encoding caveolin-1-GFP (22) or Lifeact-GFP (Ibidi, GmbH, Planegg/Martinsried, 442 443 Germany) was performed as described in (64). Briefly, cells were trypsinized and resuspended in Ingenio Solution (Mirus) containing plasmid DNA (10 $\mu g/10^6$ cells) in a 4-mm cuvette 444 (CellProjects). Cells were then electroporated at 300 V and 450 µF with one pulse of a GenePulser 445

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

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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.

458

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.

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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 °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 $(2.10^{6} \text{ cells/mL})$ 477 as described previously (42). Briefly, chambers were coated with 10 µg/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 °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 of the chamber (h_{max}) . In contrast, the pillar excludes fluorescence, which therefore reflects the 485 486 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 $\alpha = (I_{max})$ $-I_{\min}/h_{\max}$, integration of the fluorescence intensity over the cell area provided the cell volume 489 490 (V_{cell}):

 $\mathbf{V}_{\text{cell}} = \iint_{x,y} \frac{Imax - Ix, y}{\alpha} \, dx \, dy$

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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. Vassilopoulos, our collaborator. Briefly, cells were rinsed three times with Ringer's buffer with 496 497 Ca^{2+} and then briefly subjected to a concentration of 0.5 mg/mL poly-L-lysine diluted in Ca^{2+} -free Ringer's buffer (Sigma–Aldrich). Poly-L-lysine was removed by washing with Ca²⁺-free Ringer's 498 buffer. The coverslips were immersed in KHMgE buffer at 37 °C before sonication (Vibra-Cell 499 500 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% OsO4, 1.5% tannic acid and 1% uranyl acetate before 503 dehydration via successive ethanol baths, which was ultimately substituted with hexamethyldisilazane (HMDS) (#C16700-250; LFG Distribution). 504

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 incubated with a NaBH₄ solution to inactivate aldehydes. The membranes were finally fixed with 508 509 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 chamber of a metallizer (ACE600, Leica Microsystems). Once under a high vacuum (>10⁻⁵ mBar), 511 512 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 transmission electron microscope operating at 80 kV (CM120, Philips), and images were recorded 517 with a Morada digital camera (Olympus). The images were processed by ImageJ software to adjust 518 519 brightness and contrast and are presented in reverse contrast.

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521 RhoA ADP-ribosylation assay

HUVECs were seeded at a density of 27,000 cells/cm² transfected with siCTRL, siCAV1 and 522 523 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 MgCl₂ and cOmplete protease inhibitor EDTA-525 free (Roche), pH 7.5) and passed through a 27G syringe 20 times. Cell lysates were collected by centrifugation at 12,000 g for 10 min, and the protein concentration was determined by BCA assay 526 527 (Thermo Fisher Scientific). The reaction was carried out by incubating 20 µg of cell lysate with 2 µg ofExoC3 and 10 µM 6-biotin-17-NAD⁺ (BioLog) at 37 °C for 30 min. The reaction was 528 529 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 subjected to 12% SDS–PAGE, and the proportion of ADP-ribosylated (i.e., biotin-ADPr-RhoA) 531 532 RhoA in the sample was measured by Western blotting using streptavidin–peroxidase.

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

535 Plasma membrane spheres (PMS) were generated via a protocol adapted from (47). Cells were grown to 60-80% confluence on gelatine-coated 100-mm dishes and incubated for 6-8 h in PMS 536 537 buffer [1.5 mM CaCl₂, 1.5 mM MgCl₂, 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 aspirated using a casein-passivated micropipette connected to a piezo-stage (PI, Karlsruhe, 539 Germany) for manipulation and to a hydrostatic aspiration control system (66). Micropipettes were 540 541 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 a 60X water objective mounted on a confocal microscope (Nikon TE2000 inverted microscope) 545 (67). To pull tethers from PMSs, we coated streptavidin beads (3 µm in diameter, Spherotech) with 546 547 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 μ 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 to the intercept of the linear regression (f vs. $\sqrt{\sigma}$). To estimate the bending rigidity for PMSs 557 obtained from membranes of cell treated differently (siCRTL, siCAV1 and siPTRF cells), the data 558 559 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.

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562 Single-Molecule Localization Microscopy of F-actin

For single-molecule localization microscopy (SMLM) of F-actin, we used 2D STORM. HUVECs 563 564 were cultured on 1.5 high-performance coverslips coated with 10 µg/mL fibronectin for 2 h at room 565 temperature. Soluble G-actin was pre-extracted (0.25% Triton, 0.1% glutaraldehyde in 80 mM PIPES, 5 mM EGTA, and 2 mM MgCl₂, pH 6.8) for 30 s at 37 °C. The cells were then treated with 566 glutaraldehyde (0.25% Triton, 0.5% glutaraldehyde in 80 mM PIPES, 5 mM EGTA, 2 mM MgCl₂, 567 568 pH 6.8) for 10 min at 37 °C. Glutaraldehyde was quenched for 7 min at RT (0.1% NaBH₄ in PBS). Saturation was reached after 1 h at RT (0.22% gelatine and 0.1% Triton X-100 in PBS, pH 7.3). 569 Actin filaments were stained overnight at 4 °C with phalloidin-AF647 (500 nM in PBS). Cells were 570 then placed in switching buffer freshly prepared before imaging [50 mM Tris, 10 mM NaCl, 10% 571 572 glucose, 0.5 mg/mL glucose oxidase, 50 µg/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 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, Germany). Localization of individual molecules was determined using a peak mask size of 15-pixel 577 578 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.

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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 images to a maximum detection count of 10 and applied a line enhancement filter to suppress short 586 apparent discontinuities by modifying a previously described approach (70). Specifically, the 587 maxima of the mean intensities along line profiles at 15 equally spaced orientations around each 588 589 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 using Otsu thresholding. The obtained masks were subdivided into putative filament segments by 592 593 defining sections of equal length along the mask's morphological skeleton and assigning each pixel to its nearest segment (the chosen length was 20 px or approximately 0.4 μ m). The final actin filament segmentation was performed considering only segments that: 1) were not morphologically connected to at least one other segment, to exclude non-filament detections, or 2) exhibited a mean detection count lower than the super-resolution image Otsu threshold. Finally, the actin meshwork was obtained as the connected components of the inverted actin filament segmentation mask.

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600 Physical modelling

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

$$F_d = 2\sigma - \frac{T}{R},\tag{1}$$

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:

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

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 609 temperature, and κ is the effective bending rigidity of the cell membrane. While rigorously derived 610 611 for a pure lipid membrane, we assumed that Helfrich's law is applicable to describe the relationship between the effective membrane tension σ acting on TEMs and the observed projected surface in 612 our cells. Thus, the parameter κ in Eq. 2 is an *effective* bending rigidity that accounts for the role 613 614 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 the remaining caveolae can be described through modification of the effective parameters σ_0 and 616 617 к. As discussed by (21), the effective line tension is not a constant; it increases with time due to the 618

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

$$2\sigma_0 R \exp\left(-\frac{NR^2}{R_c^2}\right) - \alpha t = \mu R^2 \frac{dR}{dt},$$
(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):

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

$$\kappa \approx \frac{k_B \hat{T}}{8\pi} \frac{A_{\text{cell}}}{N A_{max}}.$$
(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 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:

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

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

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637 *Fit of the initial opening speed*

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

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

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

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

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 :

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

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	<i>σ</i> ₀ (μN/m)	A _{max} (µm²)	N (average)	(<i>N</i> ∙ <i>A</i> _{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 our experimental data using Eq. 9. The TEM maximum area, A_{max} , is the median not the average 652 653 value because the median is a more robust estimator in the presence of a few extremely large values. As discussed in the text, the variations in the bending rigidity are roughly proportional to variations 654 in $(N \cdot A_{\text{max}})^{-1}$, where N is the average number of TEMs opening simultaneously and A_{max} is the 655 656 TEM maximum area.

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658 Statistical analysis

Statistical tests were performed using the R Software v4.2.1(R Foundation for Statistical 659 660 Computing, Vienna, Austria. https://www.R-project.org/), along with packages lme4 (1.1-30)(72), ImerTest (v3.1-3)(73), emmeans (v1.8.0) (https://CRAN.R-project.org/package=emmeans), except 661 662 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 663 664 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 665 mixed-effect logistic regression. Continuous measurements (e.g. TEM area) were investigated with 666 a Gamma log-link function in a mixed-effect framework. Contrasts between multiple pairs of 667 categorical predictors were calculated through estimated marginal means with Tukey's correction. 668 P-values were calculated as indicated in the respective figure legends. P-values were considered 669 670 statistically significant at P ≤ 0.05 . Significance levels are indicated as follows: ns: not significant if *P* >0.05; * : *P* <0.05; **: *P* <0.01; ***: *P* <0.001; ****: <0.0001. 671 672

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675 H2: Supplementary Materials

- 676
- Fig. S1. CAV1^{+/+} mice susceptibility to EDIN-B in Staphylococcal septicaemia
- 678 Fig. S2. Controls of siRNAs and RhoA ADP-ribosylation efficacies.
- 679 Fig. S3. Caveolin-1 and cavin-1/PTRF depletion increases TEM formation in both ExoC3- and
- 680 EDIN-treated cells.
- 681 Fig. S4. Depletion of the caveolar accessory component EHD2 increases the TEM tunnel
- 682 formation
- Fig. S5. Caveolin-1 controls the TEM opening speed and maximum size.
- 684685 References and Notes
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951 FIGURES AND TABLES

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

954 (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 955 units per mouse (CFU/m), of LUG1799 wild-type S. aureus, which is positive for edinB (WT 956 *edinB*), and the isogenic strain LUG1840 I, in which the *edinB*-encoding gene ($\Delta edinB$) is 957 deleted. (A) Comparative analysis of CAV1^{-/-} and CAV1^{+/+} mouse susceptibility to S. aureus 958 bacteraemia. Groups of CAV1^{-/-} and CAV1^{+/+} mice were challenged via intravenous injection of 959 5×10^6 CFU/mouse (n=10 CAV1^{+/+} mice vs. n=9 CAV1^{-/-} mice), 5×10^7 CFU/mouse (n=7) 960 CAV1^{+/+} mice vs. n=9 CAV1^{-/-} mice) or 5×10^8 CFU/mouse (n=7 CAV1^{+/+} mice vs. n=8 CAV1⁻ 961 ^{/-} mice). After administering a dose of 5×10⁷ CFU/mouse, we recorded a significant increase in 962 CAV1^{-/-} susceptibility to infection compared with that of the CAV1^{+/+} littermates. Log-rank test 963 (Mantel–Cox), P < 0.0011 at 5×10⁷ CFU/mouse (1 experiment). (**B**) Lethal dose 50 (LD₅₀) of 964 WT *edinB* or $\Delta edinB$ strains administered to mice. Mice were injected with 2.5×10⁷ CFU/mouse 965 966 $(n=12 \text{ mice infected with either WT edinB or } \Delta edinB \text{ strains, } 2 \text{ experiments})$. There were no 967 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 968 969 CAV1^{-/-} mice to bloodstream infection triggered by either the WT or $\Delta e dinB$ strains. Mice were injected with 2.5×10^7 CFU/mouse (*n*=17 and 18 mice infected with WT and $\Delta edinB$ strains, from 970 971 2 experiments). Log-rank test (Mantel-Cox) showed a significantly higher susceptibility of 972 CAV1^{-/-} mice to WT *edinB* than to $\triangle edinB$ (*P*=0.0123).

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

975 (A) Confocal spinning disk images showing the actin cytoskeleton of HUVECs treated with an 976 siRNA control (siCTRL) and siRNA against caveolin-1 (siCAV1) and cavin-1 (siPTRF) and left 977 untreated (Control) or treated for 24 h with C3 exoenzyme (ExoC3). Cells were stained with 978 phalloidin-TRITC. Arrowheads show the presence of stress fibres in the absence of ExoC3 979 treatment. Stars show examples of TEM tunnels surrounded by thick actin cables. Scale bar, 20 µm. 980 (B) Histograms show the percentages of ExoC3-treated cells displaying at least one TEM (n=1,400)981 cells per condition in 8 independent experiments). Graph shows technical replicates pooled 982 together. Error bars show normal asymptotic 95% confidence intervals (CI). Data analysis with 983 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 984 985 siCAV1 and siCTRL vs siPTRF conditions. No difference was recorded between siCAV1 and siPTRF conditions. 986

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

(A) Confocal spinning disk images of HUVECs stained with phalloidin-FITC and selected 989 perimeters (yellow line) in the absence (- ExoC3) or presence of ExoC3 (+ ExoC3). Scale bar, 20 990 991 μm. (**B**) Schematic representation of the device used to define the volume of a cell by measuring 992 the intensity of fluorescence exclusion. Briefly, from the top to the bottom, i) side view of the 993 chamber in which a cell adheres to a coverslip. The PDMS pillar sustains the ceiling (grey), and the 994 maximal height of the chamber hmax (background) is known. The siCTRL-, siCAV1- or siPTRF-995 transfected HUVECs were seeded in the chamber and remained either untreated or treated with 996 ExoC3. High molecular weight dextran-FITC (green) was added to the chamber being and was 997 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 Imax and Imin yield values of maximum and minimum fluorescence intensities. Values for cell volume (V_{cell}) were obtained by integrating the fluorescence intensities 1000 1001 $h_{\text{max}} - h_{x,y}$ 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=2161008 and n=308 cells after siCTRL ± ExoC3 treatment, n=197 and n=266 cells after siCAV1 ± ExoC3 1009 treatment and n=152 and n=157 cells after siPTRF \pm ExoC3 treatment; 3 independent experiments. (**B-D**). Graph shows technical replicates pooled together. The data were analysed with a mixed-1010 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 siRNA treatment, ****P<0.0001, **P<0.01, *P<0.05 and ns, not significant. 1013

1014

Fig. 4. Inhibition of RhoA decreases caveolae density on the plasma membrane and loosens 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 μ m; high 1024 magnification, 200 nm; ExoC3, 5 μ m; 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 μ m. (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 HUVECs transfected with Lifeact-GFP expression plasmid and siCTRL, siCAV1 or siPTRF 1040 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. (B) Boxplot shows the distribution of TEMs, the maximal and median area values in HUVECs 1045 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 independent experiments. Graph shows technical replicates pooled together. Statistical data 1049 1050 analysis using a mixed-effect generalized linear model with Gamma log-link function, random intercept, and Tukey's correction for multiple comparisons. ****P<0.0001, **P<0.01 and ns, non-1051 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 tc 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 of 7 cells per treatment; >3 independent experiments. (D) The graph shows variations in mean 1057 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 > 22TEMs per condition from 4 independent experiments. 1061

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

(A) Confocal spinning disk image of a HUVEC displaying a calcein-AM-positive attached plasma 1064 membrane sphere (PMS) (arrowhead). Scale bar, 10 µm. (B) Schematic representation of the device 1065 used for measuring membrane mechanical parameters. It shows micropipette aspiration (grey) of a 1066 1067 PMS (blue) and a tube pulled from the PMS through a bead bound to the PMS and trapped with an optical tweezer (purple). Increasing the aspiration pressure in the pipette allowed a progressive 1068 increase in the PMS membrane tension. (C) Confocal images show examples of calcein-AM-1069 1070 positive PMSs prepared from siCTRL-, siCAV1- or siPTRF-transfected cells during micropipette aspiration. Scale bars, 2 μ m. (**D**) the Force required to pull membrane tubes rescaled to ((f-f₀)²/8 π ²) 1071 1072 (in pN²), which is a function of the membrane tension (mN/m) for different siCTRL, siCAV1 and 1073 siPTRF treatments. The force f₀ was measured for each tube as the force when the membrane 1074 tension vanishes. The bending rigidity κ (in k_BT) 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

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

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

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

1084 1085 SUPPLEMENTARY MATERIALS

Fig. S1. CAV1+/+ mice susceptibility to EDIN-B in Staphylococcal septicaemia.

1087 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-1088 forming units per mouse (CFU/m). Comparative analysis of CAV1^{+/+} mouse susceptibility to 1089 bloodstream infection with either the WT edinB or $\Delta edinB$ strain. Mice were injected with 5×10^6 1090 1091 CFU/m (n=5 mice infected with the WT *edinB* or $\Delta edinB$ strain; 1 experiment), 107 CFU/m (n=17) 1092 and n=19 mice infected with the WT *edinB* or $\Delta edinB$ strain, respectively; 3 experiments), 5×10^7 1093 CFU/m (n=19 or n=18 mice infected with the WT *edinB* or $\Delta edinB$ strain; 3 experiments), and 1094 5×10^8 CFU/m (n=4 or n=5 mice infected with either the WT or *\Delta edinB* strain, respectively; 1 1095 experiment). There were no significant differences in the kinetics of survival between the WT and 1096 △edinB-infected animals, as determined by log-rank test (Mantel-Cox).

1097

1098 **Fig. S2. Controls of siRNAs and RhoA ADP-ribosylation efficacies.**

1099 (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 1100 1101 GAPDH (IB: GAPDH) was used as the loading control. Immunoblots are representative of 3 1102 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-1103 1104 GAPDH antibody (IB: GAPDH) shows equal loading. The graph shows caveolin-1 and cavin-1105 1/PTRF levels normalized to the level of GAPDH (3 independent experiments). (C) Western blots 1106 show the fraction of ADP-ribosylated RhoA in cells that were resistant to a second round of in vitro 1107 ADP-ribosylation with biotin-NAD+. Western blot shows the fraction of ADP-ribosylated RhoA 1108 in vitro as detected with streptavidin-peroxidase (IB: Biotin-ADPr) i.e. not modified during cell 1109 intoxication. Immunoblotting with an anti-RhoA antibody (IB: RhoA) revealed equal RhoA protein 1110 levels. Immunoblotting with an anti-GAPDH antibody revealed equal protein loading. Blots are 1111 representative of n=3 independent experiments. The graph shows the mean values of the blot signal 1112 intensities \pm s.ds. from n=3 independent experiments. Values correspond to the signal intensities of 1113 biotin-tagged ADP-ribosylated RhoA (Biotin-ADPr) normalized to those of RhoA and GAPDH IB 1114 signals. Values are expressed as a percentage compared with the intensity of the siCTRL-1115 transfected untreated cells, which was set to 100%.

1116

Fig. S3. Caveolin-1 and cavin-1/PTRF depletion increases TEM formation in both ExoC3-1118and EDIN-treated cells.

1119 (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 1120 1121 a factor of 1 through those cells with 40 TEMs (y-axis). HUVECs were transfected with siCTRL, 1122 siCAV1 or siPTRF before treatment with ExoC3. The graph includes the geometric means (NTEM) 1123 of the density of TEMs per cell within the whole cell population \pm s.d. Graph shows technical 1124 replicates pooled together. Statistical data analysis using a mixed-effect linear model, with random 1125 intercept shows significant differences between values of N_{TEM}, ****P<0.0001. Data from n=1,400 1126 cells per condition from 7 independent experiments. (B) Histograms show the percentages of EDINtreated cells with at least one TEM (n=986 cells per treatment in 5 independent experiments). Error 1127 1128 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 1129 1130 and the siCTRL vs. siPTRF groups. No difference was recorded between the siCAV1 and siPTRF

groups. (C) The graph shows the distribution of HUVECs between classes as defined by TEM 1131 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. Statistical data analysis was based on a mixed linear model with a random intercept and fixed effects 1136 and revealed significant differences between N_{TEM} values, ****P<0.0001 (n >986 cells per 1137 1138 condition from 5 independent experiments).

1139

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

(A) Immunoblots show EHD2 cellular levels (IB: EHD2) in total cellular extracts of HUVECs 1141 transfected with siCTRL or siEHD2 and then treated for 24 h with ExoC3. Immunoblot GAPDH 1142 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 using Tukey's HSD test, ****P<0.0001 for the siCTRL versus siEHD2 groups. (C) The graph 1147 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 through those cells with 40 TEMs/cell (y-axis). HUVECs were transfected with siCTRL or siEHD2 1150 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 on a mixed linear model with a random intercept and fixed effects and revealed significant 1153 differences between N_{TEM} values, ****P<0.0001 (n= 1000 cells per condition from 5 independent 1154 1155 experiments).

1156

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

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

1162

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Glass coverslip





- ExoC3

SICTRL SICAV

3000

2000

1000

0

Volume µm³

0 SICTRI sicav¹ + ExoC3

Volume µm³

RF

, siP

3000

2000

1000

Figure 4





Α











Figure S1



Figure S2



Α





A ExoC3 treatment Figure S3







Figure S4



B ExoC3 treatment



C ExoC3 treatment



Figure S5

