

 Open access • Journal Article • DOI:10.1038/S41586-018-0466-7

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Guy Eelen, Guy Eelen, Charlotte Dubois, Anna Rita Cantelmo ...+43 more authors

Institutions: Katholieke Universiteit Leuven, Sun Yat-sen University, Lille University of Science and Technology, Max Delbrück Center for Molecular Medicine ...+9 more institutions

Published on: 29 Aug 2018 - Nature (Nature Publishing Group)

Topics: Glutamine synthetase, Glutamine, Angiogenesis, Endothelial stem cell and Protein palmitoylation

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ROLE OF GLUTAMINE SYNTHETASE IN ANGIOGENESIS

BEYOND GLUTAMINE SYNTHESIS

Guy Eelen^{1-3*§}, Charlotte Dubois^{1,3*}, Anna Rita Cantelmo^{1,3&}, Jermaine Goveia^{1,3}, Ulrike Brüning^{1,3§}, Michael DeRan⁴, Gopala Jarugumilli⁴, Jos van Rijssel⁵, Giorgio Saladino⁶, Federico Comitani⁶, Annalisa Zecchin^{1,3}, Susana Rocha⁷, Rongyuan Chen², Hongling Huang^{1,3†}, Saar Vandekeere^{1,3}, Joanna Kalucka^{1,3}, Christian Lange^{1,3‡}, Francisco Morales-Rodriguez^{1,3}, Bert Cruys^{1,3}, Lucas Treps^{1,3}, Leanne Ramer^{1,3#}, Stefan Vinckier^{1,3}, Katleen Brepoels^{1,3}, Sabine Wyns^{1,3}, Joris Souffreau^{1,3}, Luc Schoonjans^{1,3}, Wouter H. Lamers⁸, Yi Wu⁹, Jurgen Haustraete^{10,11}, Johan Hofkens⁷, Sandra Liekens¹², Richard Cubbon^{1,3,¶}, Bart Ghesquière¹³, Mieke Dewerchin^{1,3}, Francesco L. Gervasio^{6,14}, Xuri Li^{2§}, Jaap D. van Buul⁵, Xu Wu⁴ & Peter Carmeliet^{1-3§}

(1) Center for Cancer Biology, University of Leuven, Leuven, B-3000, Belgium; (2) State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China; (3) Center for Cancer Biology, VIB, Leuven, B-3000, Belgium; (4) Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129, USA; (5) Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, 1066CX Amsterdam, the Netherlands; (6) Department of Chemistry, University College London, London WC1E6BT, UK; (7) Molecular Imaging and Photonics, University of Leuven, Leuven, B-3000, Belgium; (8) Tytgat Institute for Liver and Gastrointestinal Research, Academic Medical Center University of Amsterdam, 1105BK Amsterdam, The Netherlands; (9) Center for Cell Analyses and Modelling, University of Connecticut Health Centre, Farmington 06032, USA; (10) Inflammation Research Center, VIB, Ghent, B-9000, Belgium; (11) Department of Biomedical Molecular Biology, Ghent University, Ghent, B-9000, Belgium; (12) Department of Microbiology and Immunology, University of Leuven, Leuven, B-3000, Belgium; (13) Metabolomics Core Facility, Center for Cancer Biology, VIB, Leuven, B-3000, Belgium; (14) Institute of Structural Molecular Biology, University College London, London WC1E6BT, UK. Present affiliations: (&) Université de Lille, INSERM U1003, Physiologie Cellulaire, F-59000 Lille, France; (§) Max-Delbrück-Center for Molecular Medicine, 13092 Berlin, Germany ; (†) Immunology Department, St. Jude Children's Research Hospital, Memphis 38105, USA; (‡) DFG-Research Center for Regenerative Therapies, Technical University Dresden, 01307 Dresden, Germany; (#) Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby V5A 1S6, Canada; (¶) Division of Cardiovascular and Diabetes Research, Multidisciplinary Cardiovascular Research Centre, University of Leeds, Leeds LS29JT, U.K. *equal contribution; §co-corresponding author

Editorial correspondence: P. Carmeliet & G. Eelen phone: 32-16-373204

e-mail: peter.carmeliet@kuleuven.vib.be

Xuri Li phone: 0086-20-87331815

e-mail: lixr6@mail.sysu.edu.cn

42 **Glutamine synthetase (GS) converts glutamate and NH_4^+ to glutamine. GS is**
43 **expressed by endothelial cells (ECs), but surprisingly shows negligible glutamine**
44 **synthesizing activity at physiological glutamine levels. Nonetheless, genetic loss of**
45 **GS in ECs impairs vessel sprouting during vascular development, while**
46 **pharmacological GS blockade suppresses angiogenesis in ocular and inflammatory**
47 **skin disease, only minimally affecting healthy adult quiescent ECs. This relies on**
48 **inhibition of EC migration but not proliferation. Mechanistically, GS knockdown**
49 **(GS^{KD}) reduces membrane localization and activation of the GTPase RHOJ, while**
50 **activating other Rho GTPases and Rho kinase (ROCK), thereby inducing actin**
51 **stress fibers and impeding EC motility. ROCK inhibition rescues the GS^{KD} EC**
52 **migratory defect. Notably, GS is auto-palmitoylated and interacts with RHOJ to**
53 **sustain RHOJ palmitoylation, membrane localization and activation. These findings**
54 **highlight a novel molecular activity for GS, in addition to its glutamine synthesizing**
55 **activity, in EC migration during pathological angiogenesis.**

56 Endothelial cells (ECs) line the lumen of blood vessels. Emerging evidence reveals that
57 EC metabolism controls vessel sprouting (angiogenesis)¹⁻³. While glutamine catabolism in
58 ECs was recently characterized⁴, it remains undetermined if glutamine anabolism controls
59 angiogenesis *in vivo*. Glutamine is a carbon and nitrogen donor for biomolecule production
60 and is involved in redox homeostasis. Most cells take up glutamine and thus do not need
61 to synthesize it. Nonetheless, certain cell types express glutamine synthetase (GS; also
62 called glutamate-ammonia ligase; *GLUL*), the enzyme capable of *de novo* glutamine
63 production from glutamate and ammonia in an ATP and $\text{Mg}^{2+}/\text{Mn}^{2+}$ requiring reaction. GS
64 serves also another biochemical function, i.e. ammonia clearance, but this is best
65 described for hepatocytes, astrocytes and muscle. ECs also express GS⁵, though its role
66 and importance in angiogenesis remain puzzling, given that ECs are exposed to high
67 plasma glutamine levels. Global GS deficiency causes embryonic lethality, presumably

68 due to the inability to detoxify ammonia⁶. *GS* deficiency in humans is extremely rare and
69 leads to multi-organ failure with infant death⁷. If and how *GS* affects angiogenesis has
70 never been analyzed. Here we characterized the role and importance of *GS* in vessel
71 sprouting.

72 **VESSEL SPROUTING REQUIRES ENDOTHELIAL *GS***

73 We checked *GS* expression in endothelial cells of the retinal microvasculature with a
74 genetic *GS* reporter mouse (*GS*^{+/GFP} mice with a nucleus-targeted *GFP-lamin A* fusion
75 reporter transgene in the *GS* ORF of one allele⁶). GFP tracing in the postnatal day 5 (P5)
76 retinal plexus, co-stained with the endothelial cell marker Isolectin B4 (IB4; red), revealed
77 endothelial expression of GFP (and thus of *GS*) in the microvasculature (Fig. 1a).

78 Human umbilical venous endothelial cells (further referred to as “ECs”) expressed *GS* to
79 similar levels as human colon ECs, liver ECs, human umbilical arterial ECs and blood
80 outgrowth ECs (BOECs), but to a lower level than lung ECs (Extended Data Fig. 1a).
81 However, *GS* expression in ECs or isolated mouse liver ECs (mLiECs) was lower than in
82 HEPG2 hepatocellular carcinoma cells or astrocytes (Extended Data Fig. 1a-c), known to
83 highly express *GS*. Glutamine withdrawal (below physiological concentration of 0.6 mM)
84 increased *GS* protein levels in ECs (Fig. 1b; Extended Data Fig. 1b), as previously
85 documented for other cell types⁸.

86 We intercrossed *GS*^{lox/lox} mice with two different EC-specific tamoxifen inducible Cre driver
87 lines, i.e. *VE-cadherin(PAC)-Cre*^{ERT2} and *Pdgfb-Cre*^{ERT2} mice to obtain respectively
88 *GS*^{vECKO} and *GS*^{pECKO} mice. Correct recombination of the loxed *GS* allele was confirmed
89 (Extended Data Fig. 1d-e) and caused an average 84% reduction of *GS* mRNA levels in
90 mLiECs isolated from *GS*^{vECKO} mice (Fig. 1c). In the neonatal retina, vascular plexi in P5
91 *GS*^{vECKO} mice showed hypobranching and reduced radial expansion, whereas vessel
92 coverage by NG2⁺ pericytes and vessel regression (number of empty collagen IV⁺

93 sleeves) were unaffected (Fig.1d-h, Extended Data Fig. 1f,g). However, the number of
94 filopodia at the vascular front and of distal sprouts with filopodia, both parameters of EC
95 migration, was lower in GS^{vECKO} pups (Fig 1i-j). Furthermore, the complexity of the
96 vasculature at the utmost leading front of the plexus was decreased as determined by
97 counting the number of branches in distal sprouts (Extended Data Fig. 1h). In contrast,
98 quantification of $IB4^+ EdU^+$ cells revealed no difference in the number of proliferating ECs
99 (Fig. 1k-m; Extended Data Fig. 1i). Hypobranching was also observed in the dorsal dermal
100 blood vasculature in E16.5 GS^{vECKO} embryos (Fig. 1n-r). A similar retinal phenotype was
101 observed in GS^{pECKO} mice (Extended Data Fig. 1j-m). Thus, loss of endothelial GS causes
102 vascular defects by impairing EC migration but not proliferation.

103 The retinal vascular defect restored over time (Extended Data Fig. 1n-u) and at 6 weeks,
104 GS^{vECKO} animals (with GS deleted in ECs at P1-P3) did not show overt vascular defects
105 (Extended Data Fig. 1v-ag). GS^{vECKO} animals gained normal body weight, and blood
106 biochemistry and hematological profiles were normal at 6 weeks (Extended Data Table 1).
107 Vascular restoration may relate to the possibility that homozygous mutant ECs were
108 outcompeted over time by residual wild type ECs, in which recombination did not occur (as
109 documented in mice with endothelial loss of other key metabolic genes⁹) or because of
110 other compensatory adaptations. Alternatively, the results raise the question if the effect of
111 endothelial GS loss may be larger in growing (motile) ECs during vascular development
112 than in quiescent (non-motile) ECs during adulthood in healthy conditions.

113 We then explored if pharmacological blockade of GS with methionine sulfoximine (MSO),
114 which irreversibly blocks its catalytic activity, reduced pathological angiogenesis. First, in
115 the oxygen-induced model of retinopathy of prematurity (ROP)^{2,3}, treatment of pups with
116 MSO reduced the formation of pathological vascular tufts (Fig. 2a-c), while modestly
117 increasing the vaso-obiterated area (Fig. 2d and Extended Data Fig. 1ah-ai). Second, we
118 used the corneal micro-pocket assay (CPA) in mice with slow-release basic fibroblast

119 growth factor (bFGF) containing pellets as a model of corneal neovascularization.
120 Inclusion of MSO in the pellet reduced formation of new CD31⁺ blood vessels in the
121 otherwise avascular cornea (Fig. 2e-g). Finally, we used the imiquimod-based mouse
122 model of inflammation-driven skin psoriasis and found a remarkable dose-dependent
123 reduction of the CD105⁺ EC area upon topical treatment of the affected skin with MSO
124 (Fig. 2h-l). Thus, pharmacological GS blockade inhibits pathological angiogenesis in the
125 inflamed skin and in several eye disorders.

126 **SILENCING GS REDUCES EC MIGRATION**

127 We then used GS knockdown (GS^{KD}) ECs (shRNA-mediated; >80% silencing; Extended
128 Data Fig. 2a) in *in vitro* spheroid sprouting assays to assess vessel sprouting. GS^{KD}
129 reduced the number of sprouts per spheroid and the total sprout length (Fig. 3a,b,e,f). Re-
130 introduction of a shRNA resistant GS (rGS^{OE}) rescued the sprouting defect (Extended data
131 Fig. 2b-c). The sprouting defect in GS^{KD} spheroids was maintained upon mitotic
132 inactivation of ECs with mitomycin C (MitoC) (Fig. 3c-f), further suggesting an EC motility
133 defect. In agreement, at physiological glutamine levels, GS^{KD} did not affect EC proliferation
134 (Fig. 3g). The sprouting defect was also not due to reduced EC viability or increased
135 oxidative stress, or to changes in energy charge, glutathione or NADPH levels, glycolysis,
136 glucose or glutamine oxidation, or oxygen consumption (Extended Data Fig. 2d-m).

137 GS^{KD} impaired migration in scratch-wound and Boyden chamber assays, even upon MitoC
138 treatment, an effect that was rescued by re-introducing a shRNA-resistant GS (rGS^{OE})
139 (Fig. 3h-i). Furthermore, sparsely seeded GS^{KD} ECs had a reduced velocity of random
140 movement (Fig. 3j; Supplemental videos 1 and 2) and a decreased lamellipodial area (Fig.
141 3k-m). Comparable results were obtained with a second non-overlapping shRNA and a
142 GS-specific siRNA (Extended Data Fig. 2a; Extended Data Fig. 3a-e).

143 The migration defects suggested that GS^{KD} perturbed the remodeling of the actin
144 cytoskeleton, necessary for cellular motility. Notably, we detected an increase in F-actin
145 levels in GS^{KD} ECs (Fig. 3n). A role of GS in cytoskeletal remodeling was further
146 suggested by analyzing repolymerization of the actin cytoskeleton upon disruption with the
147 F-actin polymerization inhibitor latrunculin B and subsequent wash-out. Latrunculin B
148 perturbed the normal morphology of control and GS^{KD} ECs (Fig. 3o-r). After wash-out,
149 when control cells had rebuilt a normal actin cytoskeleton, GS^{KD} ECs still had higher F-
150 actin levels, mainly originating from increased numbers of stress fiber bundles (Fig. 3s-u).
151 GS^{KD} did not alter α -tubulin levels (Fig. 3v; Extended data Fig. 4a-h).

152 The increase in F-actin levels was also present in ECs, freshly isolated from MSO-treated
153 mice (Extended data Fig. 4i-k), and in confluent GS^{KD} ECs aligning a scratch wound *in*
154 *vitro* (Extended data Fig. 4l-n). Confluent monolayer GS^{KD} ECs displayed compromised
155 junctional integrity (Extended data Fig. 4o-v). Functionally, this corresponded to a
156 decrease in trans-endothelial electrical resistance (TEER) of GS^{KD} ECs *in vitro* (Extended
157 data Fig. 4w) and increased leakiness of inflamed (but not healthy) vessels *in vivo*
158 (Extended data Fig. 4x-z).

159 **GLUTAMINE PRODUCTION BY ENDOTHELIAL GS**

160 To explore whether the migration defect was attributable to reduced *de novo* glutamine
161 synthesis, we measured the glutamine synthesizing activity of GS by supplementing ECs
162 with ¹⁵NH₄Cl (Extended Data Fig. 5a). At a physiological concentration of 0.6 mM
163 glutamine or higher, the glutamine producing activity of GS was negligible, approximating
164 the level observed in ECs treated with MSO; it slightly increased only upon glutamine
165 withdrawal, presumably to compensate for the lack of available glutamine (Fig. 4a). Similar
166 results were obtained in medium containing dialyzed serum (Extended Data Fig. 5b). For
167 further details see Supplementary Discussion 1 and Extended Data Fig. 5c-n.

168 To determine if the GS^{KD} phenotype relied on the catalytic site of GS, we used previously
169 reported concentrations of MSO¹⁰, which competes with glutamate in the catalytic site of
170 GS and irreversibly blocks GS. MSO reduced EC spheroid sprouting, impaired EC
171 migration in scratch-wound assays under MitoC treatment, decreased lamellipodial area,
172 while increasing F-actin levels after latrunculin B wash-out but without affecting EC
173 proliferation (Extended Data Fig. 5o-t). Even though other (off-target) effects of
174 pharmacological GS inhibition cannot be formally excluded, MSO phenocopied the GS
175 knockdown, suggesting that the catalytic site of GS is indispensable to control EC
176 cytoskeletal homeostasis.

177 **GS INHIBITION AFFECTS RHOJ ACTIVITY**

178 Small GTPases and their effectors control F-actin levels and motility¹¹, thus we explored if
179 Rho GTPases were downstream targets of GS. We focused on RHOJ, since it is EC-
180 enriched¹², and blocking endothelial RHOJ was proposed to be a novel anti-angiogenesis
181 approach¹³. Of note, RHOJ^{KD} ECs fully phenocopied GS^{KD} ECs in terms of decreased
182 mobility and barrier function (data not shown).

183 Since RHOJ localizes to plasma and organelle membranes to become activated¹⁴ and
184 RHOJ is almost exclusively detected in the membrane fraction¹⁵, we explored if GS levels
185 regulated RHOJ's membrane localization and activity. Immunoblotting revealed that RHOJ
186 was only detectable in the membrane fraction of ECs (consistent with previous findings¹⁵),
187 and that GS^{KD} decreased the amount of RHOJ in the membrane fraction (without
188 concomitant increase in the cytosolic fraction, possibly because of proteasomal
189 degradation¹⁶) as well as the levels of active RHOJ (Fig. 4b,c). GS^{KD} did not overtly affect
190 *RHOJ* transcript levels (relative mRNA levels: 0.99 ± 0.03 in control vs 0.85 ± 0.05 in
191 GS^{KD}; $n=3$, $P = 0.0282$).

192 We also explored if GS^{KD} affected other Rho GTPases in ECs. We focused on the
193 RHOA/B/C – Rho kinase (ROCK) – myosin light chain (MLC) axis, as silencing of
194 endothelial RHOJ increases signaling of this pathway and induces aberrant F-actin stress
195 fiber formation through an as yet undefined mechanism^{13,17} (Fig. 4d). Standard GST-
196 Rhotekin pull-down assays showed that GS^{KD} increased the activity of RHOA and RHOC,
197 but not of RHOB (Fig. 4e-g). Of note, GS^{KD} , much like other stimuli, increased total RHOB
198 levels. We confirmed the increase in RHOA activity at the individual cell level with a
199 DORA-RHOA-FRET biosensor (Fig. 4h; Extended Data Fig. 6a), and observed that the
200 abnormally elevated RHOA activity in retracting lamellipodia in GS^{KD} ECs evoked more
201 numerous, but smaller and more short-lived lamellipodia (Fig. 4i), which could contribute to
202 the motility impairment. As suggested previously¹⁸, increased RHOA activity in
203 lamellipodia locally leads to actomyosin contraction through ROCK and pMLC, thereby
204 prematurely retracting the lamellipodium. Combining GS^{KD} and $RHOJ^{KD}$ did not further
205 increase RHOA activity (data not shown) confirming that RHOJ silencing by itself
206 increased RHOA activity and suggesting that GS indeed primarily acts via RHOJ to control
207 RHOA signaling.

208 Downstream of Rho GTPases, GS^{KD} and MSO-treated ECs had elevated ROCK1 and
209 ROCK2 protein levels (Fig. 4j), and enhanced ROCK activity, as determined by pMLC
210 protein levels, which were similarly induced in GS^{KD} and $RHOJ^{KD}$ ECs (Fig. 4k; Extended
211 Data Fig. 6b-n). In agreement, ROCK inhibitors (Y27632, fasudil hydrochloride and H1152
212 dihydrochloride (not shown)) rescued the GS^{KD} phenotype (Fig. 4l-o; Extended Data Fig.
213 6o-w) whereas myosin light chain kinase (MLCK) inhibitors (ML7; peptide 18) did not
214 (Extended Data Fig. 6x-aa), suggesting that MLC phosphorylation through ROCK rather
215 than MLCK is more important in mediating the GS^{KD} phenotype in ECs. Thus, GS^{KD} lowers
216 membrane localization and activity of RHOJ, while activating RHOA, RHOC, and ROCK.

217 We explored with which of these Rho GTPases GS interacted, assuming that such an
218 interaction might facilitate / be necessary for their activation, nonetheless keeping in mind
219 that RHOJ can negatively regulate the activity of the RHOA/ROCK/MLC axis^{13,17} and
220 hence that loss of a primary interaction of GS with RHOJ could indirectly explain the
221 elevated levels of RHOA/ROCK/MLC upon GS^{KD}. First, co-immunoprecipitation (co-IP)
222 assays showed interaction between endogenous RHOJ and GS (Fig. 5a). Such co-IP was
223 not observed for RHOA and RHOC (most abundant in ECs) (Extended Data Fig. 7a).
224 Second, deletion of the first 20 N-terminal amino acids in RHOJ (Δ N20-RHOJ), mediating
225 RHOJ's plasma membrane localization¹⁹, reduced the interaction with GS (Extended data
226 Fig. 7b). Third, immunoblotting showed that only RHOJ, but not RHOA or RHOC, was
227 predominantly membrane localized (Extended data Fig. 7c). Fourth, we confirmed the GS-
228 RHOJ interaction with a bimolecular fluorescence complementation approach (BiFC)
229 (Extended Data Fig. 7d,e). Based on the above data, we focused on RHOJ as most likely
230 interacting partner of GS.

231 To interact with membrane-localized (active) RHOJ, GS should be membrane localized as
232 well. Indeed, cell fractionation studies revealed that a fraction of GS was membrane
233 localized (Fig. 5b). Further evidence derives from single particle tracking data, acquired by
234 photoactivated localization microscopy imaging (SPT-PALM), combined with total internal
235 reflection fluorescence microscopy (TIRF). We traced the movement of single GS proteins
236 tagged with the photoswitchable fluorescent protein (PSFP) mEOS (GS-mEOS). Single
237 GS-mEOS particles had a lower diffusion coefficient (DF) in the TIRF region (comprising
238 the plasma membrane and the immediately adjacent cytoplasm) than free mEOS,
239 indicative of an association of GS with membrane structures (Fig. 5c; Extended Data Fig.
240 7f).

241 **PALMITOYLATION OF GS AND RHOJ**

242 Membrane localization often requires post-translational palmitoylation. We thus
243 hypothesized that GS could be palmitoylated to allow plasma membrane localization and
244 interaction with RHOJ. Therefore, we performed click chemistry with biotin-azide
245 (Extended Data Fig. 7g) on lysates from HEK293 cells overexpressing GS and treated with
246 the clickable palmitoylation probes 16C-BYA or 16C-YA. Streptavidin pull-down showed
247 clear palmitoylation of GS, as both probes labeled GS. The labeling was reduced by MSO,
248 consistent with the presumed dependency of the phenotype on the enzyme's catalytic site
249 (Fig. 5d).

250 GS was anecdotally reported previously to be palmitoylated, however without further in-
251 depth molecular / functional characterization²⁰. To determine if GS undergoes
252 autopalmitoylation, we incubated purified GS²¹ with palmitoyl-alkyne CoA (a substrate for
253 palmitoylation) in a cell-free system without any other proteins present, to demonstrate a
254 direct effect. Click chemistry revealed that increasing the dose of palmitoyl-alkyne CoA
255 resulted in increased autopalmitoylation of GS (Fig. 5e). Importantly, autopalmitoylation of
256 GS was achieved with physiological concentrations of palmitoyl-CoA (1-10 μ M) at neutral
257 pH, suggesting physiologically relevant autopalmitoylation and was confirmed with two
258 alternative methods (Supplementary Discussion 2 and Extended Data Fig. 7h-j).

259 Palmitoylation of target proteins by palmitoyl-acyl transferases (PATs) is a two-step
260 reaction, requiring first autopalmitoylation of the PAT, and thereafter, transfer of the
261 palmitoyl group to the target protein. We hypothesized GS to have a similar activity profile
262 (Supplementary Discussion 3) and explored if GS was involved in palmitoylation of RHOJ.
263 Even though RHOJ's cysteines at position 3 (C3) and 11 (C11) were *in silico* predicted to
264 be high fidelity palmitoylation sites (screened with SwissPalm²², data not shown),
265 palmitoylation of RHOJ has been poorly documented (except in a few studies^{23,24}).
266 Interestingly, RHOJ's membrane localization and activity were reduced by treatment of
267 ECs with the pan-palmitoylation inhibitor 2-bromopalmitate (2BP) and by point mutating

268 cysteines C3 and C11 (Fig. 5f; Extended Data Fig. 7k-t), providing initial evidence that
269 RHOJ can be palmitoylated in ECs. Using the palmitoylation probe 17-ODYA (Fig. 5g) or
270 an acyl-resin-assisted capture (acyl-RAC; Extended Data Fig. 7u), we found a reduction in
271 the levels of palmitoylated RHOJ upon blocking GS, consistent with a model whereby GS
272 sustains palmitoylation of RHOJ.

273

274 **DISCUSSION**

275 Surprisingly, we found a glutamine synthesizing-independent activity for GS in regulating
276 EC motility, even though we cannot formally exclude a possible contribution of minimal
277 levels of glutamine production by GS to the observed phenotype. Indeed, GS regulates
278 RHOJ signaling in cell motility as shown by several lines of evidence. First, a fraction of
279 GS is present in EC membranes, where active RHOJ resides. Second, GS interacts with
280 RHOJ in ECs in co-IP experiments (though this interaction can be direct / indirect). Third,
281 GS^{KD} reduces RHOJ's palmitoylation, membrane localization and activity in ECs. Thus,
282 since RHOJ promotes EC motility^{13,17}, the impaired migration of GS^{KD} ECs could be
283 attributed to the reduced RHOJ activity. RHOJ likely also indirectly contributes to
284 promoting EC motility through controlling the activity of the RHOA/ROCK/MLC signaling
285 pathway, known to regulate EC motility by affecting stress fiber formation^{13,17} (Extended
286 Data Fig. 7v; Supplementary Discussion 4).

287 Because purified GS seems capable of autopalmitoylation (a trademark of PAT enzymes),
288 and GS silencing lowers RHOJ palmitoylation, our data support a model, whereby GS first
289 autopalmitoylates itself and thereafter transfers the palmitoyl group to RHOJ, though we
290 cannot formally exclude that transfer of the palmitoyl group from GS to RHOJ occurs via
291 additional partners or even non-enzymatically. A possible model for GS palmitoylation is

292 described in Supplementary Discussion 5, Extended Data Fig. 8 and Extended Data Table
293 2. Also, whether the GS-RHOJ partnership is exclusive or GS interacts with other players
294 (*eg* other palmitoylated RhoGTPases such as RAC1, CDC42, RHOU or RHOV) to mediate
295 this effect on EC motility, remains outstanding. In any case, RHOJ seems to be a critical
296 target of GS, given that its silencing completely phenocopies GS inhibition in ECs.

297 Finally, GS is critical for EC motility / migration, contributing to the formation of new
298 vessels in development and disease. In contrast, ECs do not migrate when they are
299 quiescent in healthy adults, explaining why GS inhibition has no observable effects on the
300 vasculature in healthy adult mice. This renders GS an attractive disease-restricted target
301 for therapeutic inhibition of pathological angiogenesis. In agreement, the pharmacological
302 GS blocker MSO reduced pathological angiogenesis in blinding eye and psoriatic skin
303 disease (Fig. 2), which warrants further exploration of GS targeting in anti-angiogenesis.

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392 **Supplementary Information** is linked to the online version of the paper at
393 www.nature.com/nature.

394

395

396 **ONLINE CONTENT**

397 Methods and associated references, and Extended Data display items are available in the
398 online version of the paper.

399

400 **ACKNOWLEDGMENTS**

401 We acknowledge Rod Levine for supplying purified bacterial GS, Ludo Van Den Bosch
402 and Wendy Scheveneels for providing primary mouse astrocytes, Sander Trensou, Inne
403 Crèvecoeur, Sam Noppen, Lizette Van Berckelaer and Ria Van Berwaer for technical
404 assistance, Sarah-Maria Fendt, Dries Verdegem and Chris Ulens for discussions and
405 suggestions, Wesley Vermaelen, Abel Acosta Sanchez, Aleksandra Brajic, Agua Sobrino
406 and Maaike Cockx for experimental assistance, Lena-Christin Conradi, Andreas Pircher for
407 supplying materials and Els Wauters, Albert Wolthuis and Joris Jaekers for providing
408 tissues for EC isolations. HecBioSim and PRACE are acknowledged for allocation of
409 computer time. JG, ARC, CD, CL, JK, FJMR, SR and SV are supported by the FWO; AZ
410 by LE&RN/FDRS; BC by IWT; UB by a Marie Curie-IEF Fellowship; HH by an EMBO
411 longterm fellowship; JDvB by a LSBR fellowship; RC by a British Heart Foundation
412 Intermediate Clinical Fellowship; and XW by the American Cancer Society RSG, NIH/NCI
413 and NIH/NIDDK. FC, GS and FLG are supported by EPSRC; XL by the State Key
414 Laboratory of Ophthalmology, Zhongshan Ophthalmic Center at Sun Yat-Sen University,
415 and the National Natural Science Foundation of China (81330021 and 81670855). PC is
416 supported by a Federal Government Belgium grant, long-term structural Methusalem
417 funding by the Flemish Government, a Concerted Research Activities Belgium grant,
418 grants from the FWO, Foundation against Cancer and ERC Advanced Research Grant.
419 GE and MD received a Foundation against Cancer grant and GE received a FWO 'Krediet
420 aan navorsers'.

421

422 **AUTHOR CONTRIBUTIONS**

423 *Study concept and supervision:* PC; *contribution to the execution, support and analysis of*
424 *experiments, and/or advice:* GE, PC, XL, MD, LS; *experimental design:* GE, CD, ARC, JG
425 *and PC; molecular biology and in vivo experiments:* GE, CD, ARC, JG, UB, AZ, HH, SVa,

426 JK, CL, FMR, BC, LR, SVi, KB, SW, JS, LS, SL, RCh, RCu, MD; *mass spectrometry*: BG;
427 *RHO activity assays*: JvR, JDvB; *GS palmitoylation*: MDR, GJ, XW; *molecular dynamics*
428 *simulations*: GS, FC, FLG; *BiFC and SPT*: SR, JHo; *data interpretation*: GE, CD, ARC, JG,
429 RC, UB, CL, SR, LT, BC, MD, JHo, SL, BG, FLG, JDvB, XW and PC; *providing necessary*
430 *materials*: WHL, YW and JHa; *manuscript drafting*: GE and PC. All authors agreed on the
431 final version of the manuscript.

432

433 **AUTHOR INFORMATION**

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435 The authors declare no competing interests.

436 Correspondence and requests for materials should be addressed to PC
437 (peter.carmeliet@kuleuven.vib.be).

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439

440 **LEGENDS TO FIGURES**441 **FIGURE 1: EC-SPECIFIC DELETION OF GS CAUSES VASCULAR DEFECTS *IN VIVO***

442 **a**, GS expression (arrowheads) in the retinal microvasculature (co-stained with isolectin B4
443 (IB4)) of five day-old (P5) chimeric $GS^{+/GFP}$ pups (with zoom-in inset). **b**, GS protein levels
444 in HUVECs under different extracellular glutamine levels. **c**, GS mRNA levels upon
445 activation of VE-cadherin-Cre^{ERT2}. **d-g**, IB4 staining of P5 retinal vascular plexi from WT
446 (d) and GS^{vECKO} (e) mice (with zoom-in insets, A=artery, V=vein) and quantification of
447 branch points at the front of the plexus (f) and radial expansion of the plexus (g). **h**, Vessel
448 regression (area of collagen IV (Col IV)⁺ IB4⁻ vessel sleeves (% of total Col IV⁺ area)) in
449 retinas from P5 WT and GS^{vECKO} pups. **i-j**, Distal sprouts (i) and filopodia (j) at the retinal
450 vascular front. **k-m**, IB4 (gray)/EdU (cyan) double staining of P5 WT (l) and GS^{vECKO} (m)
451 retinas (arrowheads in zoom-in insets denote EdU⁺ ECs) and quantification (k) of EdU⁺
452 ECs at the front of the plexus. **n-r**, CD31-stained dermal dorsal blood vasculature in E16.5
453 WT (n,o) and GS^{vECKO} (p,q) mice with boxed regions magnified in (o) and (q) and
454 quantification of number of branch points (r). All data are mean±s.e.m; n-number
455 (individual experiments) is 2 (a,b); n-numbers (individual mice) for WT and GS^{vECKO} are: 3
456 and 3 (c); 11 and 10 (f); 10 and 7 (g); 4 and 6 (h); 18 and 22 (i); 17 and 21 (j); 12 and 22
457 (k); 5 and 15 (r), from 2 (g,h,r), 3 (f) or 4 (i,j,k) litters. ^{NS} $P>0.05$, $*P<0.05$ according to
458 Student's *t* test (c,g,h,i,j,k,r) or mixed models R statistics (f). Exact *P* values: (c) 0.0215; (f)
459 0.0141; (g) 0.0063; (h) 0.4902; (i) 0.0009; (j) 0.0484; (k) 0.3837; (r) 0.0046. Scale bars: 10
460 μm (a right), 50 μm (a left), 100 μm (l,m), 200 μm (d,e,n,p). Gel source images: see
461 Supplemental Information Fig. 1.

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464

465 **FIGURE 2: GS INHIBITION MITIGATES PATHOLOGICAL ANGIOGENESIS**

466 **a-d**, Retinal flat-mounts of retinopathy of prematurity (ROP) mice treated with vehicle (a) or
467 20 mg kg⁻¹ d⁻¹ MSO (b). Quantification of vascular tuft (c) and vaso-obiterated area (d) in
468 control and MSO-treated ROP pups. **e-g**, Quantification (e) of CD31⁺ (green) neo-vessels
469 in corneal flat-mounts from mice in corneal pocket assays (CPA) with bFGF pellets
470 (demarcated by dotted white line) with vehicle (f) or MSO (g). **h-l**, CD105 staining of
471 untreated skin (h), IMQ-treated skin (i), IMQ + low dose MSO-treated skin (j), IMQ + high
472 dose MSO-treated skin (k), and quantification of CD105⁺ area (l). All data are
473 mean±s.e.m.; n-numbers (individual mice) for control and MSO-treated are: 7 and 6 (c,d),
474 10 and 11 (e) from 3 litters (c,d) and 2 experiments (e). In (l) n=15 for control, n=22 for
475 IMQ, n=18 for IMQ + MSO low (indicated by +) and n=6 animals for IMQ + MSO high
476 (indicated by ++) from 3 experiments. ^{NS}P>0.05, *P<0.05 according to Student's *t* test
477 (c,d,e) or ANOVA with Dunnett's multiple comparisons vs IMQ (l). Exact *P* values (c)
478 0.0459; (d) 0.0145; (e) <0.0001; (l) ctrl vs IMQ: 0.0278; MSO low vs IMQ: 0.7283; MSO
479 high vs IMQ: 0.0451. bFGF: basic fibroblast growth factor; CD31: cluster of differentiation
480 31; IMQ: imiquimod; MSO: methionine sulfoximine. Scale bars: 100 μm (a,b), 200 μm (f,g),
481 75 μm (h-k).

482

483 **FIGURE 3: LOSS OF GS IMPAIRS EC MIGRATION THROUGH PERTURBED ACTIN DYNAMICS**

484 **a-f**, Control (a,c) and GS^{KD} (b,d) EC spheroids without (a,b) and with mitomycin C (MitoC)
485 (c,d) treatment and number of sprouts per spheroid (e) and total sprout length (f). **g**, [³H]-
486 Thymidine incorporation in control and GS^{KD} ECs. **h**, Wound closure upon MitoC-treatment
487 of control and GS^{KD} ECs. **i**, Boyden chamber migration for control, GS^{KD} and GS^{KD} +
488 rGS^{OE} (overexpression of a shRNA-resistant *GS* mutant) ECs, all under MitoC-treatment.

489 **j**, Velocity of sparsely seeded control and GS^{KD} ECs. **k-m**, Phalloidin (F-actin) staining of
 490 control (k) and GS^{KD} (l) ECs (arrows and white dotted lines indicate lamellipodia) and
 491 quantification of lamellipodial area (m). **n-p**, F-actin and G-actin levels in phalloidin (F-
 492 actin) – DNase I (G-actin) double-stained control and GS^{KD} ECs (n), and representative
 493 images of phalloidin-stained control (o) and GS^{KD} (p) ECs. **q-u**, Phalloidin staining of
 494 latrunculin B-treated control (q,s) and GS^{KD} (r,t) ECs at timepoint 0 (q,r) and at 1 h after
 495 latrunculin wash-out (s,t) and quantification of F-actin levels after wash-out (u). **v**, α -
 496 Tubulin levels in GS^{KD} and control ECs. All data are mean \pm s.e.m.; n-numbers
 497 (independent experiments) are: 4 (e,f), 9 (g,j), 5 (h), 6 (i,u), 7 (m) and 3 (n,v). ^{NS} $P > 0.05$,
 498 $*P < 0.05$ according to mixed models R statistics (e,f), Student's *t* test (g,h,j,m,n,u,v) or
 499 ANOVA with Dunnett's multiple comparison vs control (i). Exact *P* values (e,f) ctrl vs GS^{KD}
 500 \pm mitoC: < 0.0001 ; (g) 0.7729; (h) 0.0283; (i) ctrl vs GS^{KD}: 0.0093; ctrl vs GS^{KD} + rGS^{OE}:
 501 0.5981; (j) 0.0234; (m) 0.0352; (n) F-actin: 0.0467; G-actin: 0.584; (u) 0.0007; (v) 0.3491.
 502 AU, arbitrary units. Scale bars: 100 μ m (a-d), 10 μ m (k,l) and 20 μ m (o-t).

503

504 **FIGURE 4: ENDOTHELIAL GS REGULATES RHO GTPASE ACTIVITY**

505 **a**, Effect of glutamine and MSO on glutamine-producing activity (% enrichment in m+1
 506 glutamine and glutamate, 30 min after adding ¹⁵NH₄⁺). **b**, RHOJ, NaK ATPase (membrane
 507 marker) and GAPDH (cytosol marker) immunoblots in cytosolic (c) and membrane (m)
 508 fractions with quantification **c**, Immunoblot for active and total RHOJ with quantification
 509 (RHOJ^{KD}, beads only and irrelevant biotinylated peptide are negative controls) **d**, RHOJ's
 510 pivotal yet incompletely understood (question mark) role in EC migration/stress fiber
 511 formation. **e-g**, Immunoblots for pull-down RHOA (e), RHOB (f) and RHOC (g) activity
 512 assays with quantifications. **h**, Control and GS^{KD} ECs expressing the DORA RHOA
 513 biosensor, with quantification of whole-cell FRET startratio (mean \pm s.e.m.; control, n=12
 514 cells; GS^{KD}, n=9). Look-up table (LUT; color bar) denotes relative RHOA activities

515 (blue=low, red=high). **i**, Kymograph of DORA RHOA biosensor expressing ECs, showing
 516 abnormally short-lived lamellipodia and increased RHOA activity in retracting lamellipodia
 517 of GS^{KD} ECs (red arrowheads) (representative of 13 control and GS^{KD} cells). **j**, ROCK1,
 518 ROCK2, α -tubulin immunoblots with quantification. **k**, pMLC, total MLC and α -tubulin
 519 immunoblots (quantification see Methods section). **l**, F-actin levels after latrunculin B
 520 wash-out in ECs treated with the ROCK inhibitor Y27632. **m-o**, Effect of Y27632 on
 521 spheroid sprouting defect (m), migration defect (n), and lamellipodial area (o). Values in
 522 l,n,o are relative to untreated non-silenced control (dotted line). pMLC: phosphorylated
 523 MLC. Scale bar is 25 μ m (h). All data are mean \pm s.e.m.; n-numbers (independent
 524 experiments) are: 3 (a,e,f,m,n), 4 (c(MSO),h,k,l), 5 (o), 7 (j), 8 (c(GS^{KD}), g), 13 (b).
 525 ^{NS} $P>0.05$, [#] $P=0.05$, ^{*} $P<0.05$; ANOVA with Dunnett's multiple comparisons vs 4 mM (a),
 526 one sample *t* test (b,c,e,f,g,j,k), Student's *t* test (h,n,o), paired Student's *t* test (l) or mixed
 527 models R statistics (m). Exact *P* values (a) (Glu) 0.6 mM vs 4 mM: 0.9903; 0.025 mM +
 528 MSO vs 4 mM: 0.0968; 0.025 mM vs 4 mM: 0.1943; (Gln) 0.6 mM vs 4 mM: 0.4518; 0.025
 529 mM + MSO vs 4 mM: 0.9999; 0.025 mM vs 4 mM: 0.0143; (b) 0.0072; (c) MSO: 0.0323;
 530 GS^{KD}: 0.0095; (e) 0.053; (f) 0.1790; (g) 0.0035; (h) 0.0055; (j) ROCK1 MSO: 0.0169;
 531 ROCK1 GS^{KD}: 0.0138; ROCK2 MSO: 0.0381; ROCK2 GS^{KD}: 0.0802; (k) MSO: 0.0283;
 532 GS^{KD}: 0.0431; RHOJ^{KD}: 0.0091; (l) 0.0431; (m) GS^{KD} vs ctrl: <0.0001; GS^{KD} + Y27632 vs
 533 ctrl + Y27632: 0.5211; (n) 0.0181; (o) 0.0210. Gel source images: see Supplemental
 534 Information Fig. 1.

535

536 **FIGURE 5: GS (AUTO)-PALMITOYLATION**

537 **a**, Co-immunoprecipitation (Co-IP) of endogenous RHOJ and GS in ECs. Upper panel: IP
 538 of RHOJ; lower panel: IP for GS. **b**, Immunoblot for GS and RHOJ in cytosolic (c) and
 539 membrane (m) fractions in ECs with NaK and GAPDH as fraction markers. **c**, Diffusion
 540 coefficient (DF, in $\mu\text{m}^2\text{s}^{-1}$) of single photoswitchable fluorescent protein mEOS and mEOS-

541 fused GS (mEOS-GS) particles in the plasma membrane region of ECs acquired by SPT-
542 PALM under TIRF illumination (n=41 cells expressing mEOS and 37 expressing mEOS-
543 GS) **d**, GS immunoblotting after streptavidin pull-down of biotin-azide clicked lysates from
544 HEK-293T cells for the indicated palmitoylation probes. Input shows levels of GS
545 overexpression. **e**, Dose-effect of palmitoyl-alkyne CoA on autopalmitoylation of purified
546 GS; biotin-azide clicking and HRP-streptavidin blotting; input on Coomassie-stained gel. **f**,
547 Immunoblotting for RHOJ, NaK and GAPDH in membrane (m) and cytosolic (c) fractions of
548 control- and 2BP-treated ECs. **g**, Palmitoylation of RHOJ in GS^{KD}, MSO- and 2BP-treated
549 ECs. In-gel fluorescence for TAMRA-azide 17-ODYA (palmitoylation probe)-clicked FLAG-
550 RHOJ is shown (FLAG as loading control). 2BP, 2-bromopalmitate, pan-palmitoylation
551 inhibitor. All data are mean±s.e.m., except box and whisker (running from minimal to
552 maximal values) plots in (c); n-numbers (independent experiments) are: 2 (e), 3 (a,b,c,d,f),
553 4(g). ^{NS}*P*>0.05, **P*<0.05; Student's *t* test (c); one sample *t* test (f,g). Exact *P* values (c)
554 <0.0001; (f) 0.0264; (g) MSO: 0.0317; GS^{KD}: 0.0003; 2BP: 0.0163. Gel source images: see
555 Supplemental Information Fig. 1.

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569 **METHODS**

570 **CHEMICALS AND REAGENTS:** The GS inhibitor L-methionine sulfoximine (MSO), mitomycin C,
571 latrunculin B, oligomycin, antimycin A, carbonyl cyanide-4-(trifluoromethoxy)
572 phenylhydrazone (FCCP), 2-bromohexadecanoic acid (2-bromopalmitic acid, 2BP),
573 tamoxifen, palmitoyl-CoA agarose and α -ketoglutarate dehydrogenase were from Sigma-
574 Aldrich. 17-Octadecynoic acid (17-ODYA) was purchased from Cayman Chemical. The
575 use and/or synthesis of the other palmitoylation probes 15-hexadecynoic acid (16C-YA; a
576 palmitate-based probe that binds a broader spectrum of proteins than 16C-BYA (here
577 below), including both PATs and PAT target proteins) and 2-bromooctadec-15-ynoic acid
578 (16C-BYA; a 2-bromopalmitate-based activity-based probe that labels but also inhibits
579 palmitoyl acyltransferase (PAT) enzymes) has been described previously²⁵. The ROCK
580 kinase inhibitor Y27632 ((1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-
581 yl)cyclohexanecarboxamide) was from BioVision, fasudil hydrochloride and H1152
582 dihydrochloride are from Tocris. The MLCK inhibitors ML7-hydrochloride and peptide 18
583 were from Tocris. Collagen type 1 (rat tail) was obtained from Merck Millipore. [5-³H]-
584 glucose, [³H]-thymidine, [U-¹⁴C]-glutamine were from Perkin Elmer; [6-¹⁴C]-D-glucose was
585 from ARC. [U-¹³C]-glucose, [U-¹³C]-glutamine, [U-¹³C]-glutamate and ¹⁵NH₄Cl were
586 purchased from Cambridge Isotope Laboratories. The following primary antibodies or dyes
587 were used (dilutions for staining (ST), immunoblotting (IB), immunofluorescence (IF) and
588 immunoprecipitation (IP) are given in between brackets): *Griffonia simplicifolia* (GS)-IB₄-
589 Alexa 488 (ST 1:200), isolectin GS-IB₄-Alexa 568 (ST 1:200), isolectin GS-IB₄-Alexa 647
590 (ST 1:200), phalloidin-Alexa 488 (ST 1:100), deoxyribonuclease I-Alexa 594 (ST 1:200)
591 (Molecular Probes), anti-collagen IV (2150-1470) (IF 1:400) (Bio Rad), anti-NG2

592 Chondroitin Sulfate Proteoglycan (AB5320) (IF 1:200) (Millipore), anti-FLAG (clone M2) (IB
593 1:1,000; IP 5 $\mu\text{g ml}^{-1}$), anti-GS (clone 2B12) (IB 1:1,000; IP 2-5 $\mu\text{g ml}^{-1}$), anti-RHOJ (clone
594 1E4) (IB 1:1,000; IP 2-5 $\mu\text{g ml}^{-1}$), anti-ROCK1 (HPA007567) (IB 1:1,000), anti- α -tubulin
595 (T6199) (IB 1:1,000) (Sigma-Aldrich), anti- β -actin (13E5) (IB 1:1,000), anti-phospho-
596 Myosin Light Chain 2 (IB 1:1,000; IF 1:300) and anti-Myosin Light Chain 2 (IB 1:1,000)
597 (9776), anti-Na,K-ATPase (NaK) (3010) (IB 1:1,000), anti-RHOA (67B9) (IB 1:1,000) and
598 anti-RHOC (D40E4) (IB 1:1,000) (Cell Signaling Technology), anti-CD105/endoglin
599 (AF1320) (IF 1:50), anti-VE-cadherin (AF1002) (IF 1:50) (R&D Systems), anti-ROCK2
600 (A300-047A-T) (IB 1:500) (Imtec Diagnostics), anti-CD31 (MEC13.3) (IF 1:200), anti-
601 CD34-biotin (#553732) (IF 1:25) (BD Biosciences), anti-RHOB (sc-180) (IB 1:1,000).
602 Secondary Alexa-405, -488, -568 or -647 conjugated antibodies (1:500) were from
603 Molecular Probes; other secondary antibodies and IgG controls were from Dako. The
604 Click-iT[®] 5-ethynyl-2'-deoxyuridine (EdU) Alexa Fluor[®] 555 Imaging Kit was from
605 Invitrogen. Purified bacterial GS was a kind gift from Rod Levine (Bethesda, MD, USA).

606 **CELL CULTURE:** *HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS) AND HUMAN UMBILICAL*
607 *ARTERY ENDOTHELIAL CELLS (HUAECs)* obtained under protocol S57123 (Commission
608 Medical Ethics of UZ/KU Leuven) after written consent of the donors, were isolated as
609 previously described ^{1,2} and were routinely cultured in M199 medium (Invitrogen)
610 containing 20% FBS, 0.6 mM L-glutamine, heparin (10 U ml^{-1} ; Sigma), penicillin (100 U ml^{-1})
611 ¹), streptomycin (100 $\mu\text{g ml}^{-1}$) and endothelial cell growth factor supplements (EGCS; 30
612 mg l^{-1} ; Sigma). Cells were only used between passages 1 and 4 and all experiments were
613 performed in HUVECs from at least three different donors unless stated otherwise. Also
614 except when stated otherwise, the use of the abbreviation EC in the text refers to HUVEC.
615 *ISOLATION OF ENDOTHELIAL CELLS FROM HUMAN LUNG/LIVER/COLON MUCOSA:* Lung/liver/colon
616 mucosa specimens were obtained under protocol S57123 (Commission Medical Ethics of
617 UZ/KU Leuven) and were washed several times with phosphate buffer solution (PBS) and

618 minced with scissors prior to enzymatic digestion for 45 min. at 37 °C with
619 collagenase/dispase/DNase solution (Gibco, Life Technologies). The resulting suspension
620 was passed through a 100 μ m nylon mesh (BD Biosciences Pharmingen) to remove
621 aggregates. The harvested cells were washed, seeded on gelatin pre-coated 6-well plates
622 and cultured in complete endothelial growth medium (EGM-MV; Lonza) supplemented with
623 antibiotics. After 5-7 days, when cells reached confluency, a positive CD31 magnetic bead
624 selection was performed (CD31 MicroBead, #130-091-935, Miltenyi Biotech) according to
625 the manufacturer's guidelines and purified cells were further cultured in EGM medium.
626 *PERIPHERAL BLOOD OUTGROWTH ENDOTHELIAL CELLS (BOECs)* were established and cultured
627 as previously described ²⁶. In brief, blood samples (obtained under protocol S57123
628 (Commission Medical Ethics of UZ/KU Leuven) were diluted with PBS prior to Ficoll
629 PaquePLUS (GE Healthcare) density-gradient centrifugation at 1,000 g for 20 min at room
630 temperature. The mono-nuclear cell layer was collected, washed with PBS and
631 resuspended in EGM2 medium (PromoCell). Cells were plated in collagen-coated flasks
632 and medium was replaced every 2 days. From day 7 onwards, cells were checked for the
633 formation of colonies, which were allowed to grow up to approximately 1 cm². BOEC
634 colonies were then trypsinized and subcultured. *HEK293T AND HEPG2 CELLS* (ATCC) were
635 grown in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin
636 and 100 μ g ml⁻¹ streptomycin. When HEPG2 cells were compared directly to ECs in short
637 term stable isotope tracing experiments, they were incubated in exactly the same medium
638 as the ECs to rule out possible bias coming from the difference in media formulation. We
639 did not perform authentication of the HEK293T and HEPG2 cells. *MOUSE LIVER*
640 *ENDOTHELIAL CELLS (MLIECs)* were isolated from perfused healthy livers of control or
641 GS^{ECKO} mice. Prior to perfusion, the mice were anesthetized with Nembutal (60 mg kg⁻¹).
642 Mice were perfused with 5 ml of a water-based perfusion buffer containing 1.7 M NaCl, 84
643 mM KCl, 120 mM HEPES and 1 mM NaOH followed by 5 ml of a PBS-based digestion

644 buffer containing 0.1% collagenase II (Life Technologies), collagenase I (Life
645 Technologies), 2 mM CaCl₂, 1% antibiotic-antimycotic (Life Technologies) and 10% FBS
646 (Biochrome, Berlin, Germany) at a perfusion rate of 1 ml min⁻¹. Perfusion was considered
647 complete when the liver and mesenteric vessels were blanched and the desired amount of
648 digestion buffer (≥ 5ml) had passed through the circulatory system. Livers were dissected,
649 placed into a 50 ml conical tube with 3 ml of digestion buffer and incubated at 37 °C for
650 approximately 30 min, with regular shaking of the tubes every 5 min. After digestion, the
651 tissue was homogeneously dissociated and the reaction was stopped with 10 ml of
652 isolation buffer containing PBS + 0.1% BSA (Sigma-Aldrich). Subsequently, the cell
653 suspension was filtered through a 100 µm cell strainer and cells were washed twice with
654 isolation buffer. Finally, the ECs were isolated by magnetic bead sorting with Dynabeads
655 (CELLlection™ Biotin Binder Kit, Life Technologies, Ghent, Belgium) coated with anti-
656 mouse CD31 (eBioscience, Anti-Mouse CD31 Clone 390), according to the manufacturer's
657 instructions. Briefly, the cell suspension was incubated with the beads at room
658 temperature for 30 min in HulaMixer® Sample Mixer (Life Technologies, Ghent, Belgium).
659 Next, CD31⁺ ECs were collected by putting the tubes on a DynaMag™-50 Magnet (Life
660 Technologies) and removing the supernatant. The procedure was repeated twice to
661 remove cells debris. Finally, cells were resuspended in EGM2 medium (PromoCell) and
662 plated at the desired density on cell culture plates pre-coated with 0.1% gelatin, and grown
663 to confluency. *MOUSE ASTROCYTES* were prepared as described previously with minor
664 changes ²⁷. Briefly, spinal cords were dissected from 13-day old C57BL/6J mouse
665 embryos. Meninges and dorsal root ganglia were removed and a single cell population
666 was obtained by digestion with 0.05% trypsin in combination with gentle trituration. The
667 cell suspension was layered on a 6.2% OptiPrep™ (Axis-Shield, Oslo, Norway) cushion
668 and centrifuged at 500g for 15 min. The pellet was resuspended and the cells were plated
669 (12,000 cells cm⁻²) in L15 medium supplemented with glucose (3.6 mg ml⁻¹), sodium

670 bicarbonate (0.2%), penicillin (100 IU ml⁻¹), streptomycin (100 µg ml⁻¹) and fetal bovine
671 serum (10%). After reaching confluency, cell division was halted by treatment with cytosine
672 arabinoside (10 µM, 3 days). After 4 weeks, more than 95% of cells stained positive for
673 glial fibrillary acidic protein (GFAP; not shown). We routinely tested primary cells and cell
674 lines for mycoplasma contamination with the MycoAlert mycoplasma detection kit (Lonza,
675 LT07-418).

676 **PLASMID CONSTRUCTIONS AND LENTIVIRAL PARTICLE PRODUCTION:** cDNA for human *GS* was
677 obtained from Origene. Silent mutations were introduced to make the *GS* cDNA resistant
678 to the *GS*-specific shRNA (see below, TRCN0000045628). Point-mutated constructs were
679 generated with Stratagene's QuickChange site-directed mutagenesis kit following
680 manufacturer's guidelines. The cDNA for *RHOJ-EGFP (GFP-TCL)* was a gift from
681 Channing Der (Addgene plasmid # 23231)²³ and was used as a template to generate the
682 N-terminal truncated Δ N20-RHOJ-EGFP, lacking the first 20 amino acids and FLAG-
683 tagged RHOJ. Standard cloning techniques were used to fuse *GS* to the photoswitchable
684 fluorescent protein mEOS (pRSETa-mEos2 was a gift from Loren Looger; Addgene
685 plasmid # 20341)²⁸. The BiFC vector allowing simultaneous expression of two separate
686 cDNAs fused to EGFP subfragment 1 (N-terminal; containing amino acids 1 to 158) or
687 subfragment 2 (C-terminal; containing amino acids from 159 onwards) respectively was a
688 kind gift of Prof. Hideaki Mizuno (KU Leuven). *GS* was fused to the N-terminal
689 subfragment of EGFP and RHOJ was fused to the C-terminal EGFP subfragment to
690 generate *GS-EGFP*^{1/2}, *RHOJ-EGFP*^{2/2}. Lentiviral expression constructs were obtained by
691 cloning the respective cDNAs into pRRLsinPPT.CMV.MCS MM WPRE-vector. Validated
692 *GS*-specific (TRC clones TRCN0000045628 (used in the majority of the experiments and
693 indicated as *GS*^{KD1} in Extended Data Fig. 2a) and TRCN0000045631 (indicated as *GS*^{KD2}
694 in Extended Data Fig. 2a and only used to confirm the migration and lamellipodial defect in
695 Extended Data Fig. 3a-b) and *RHOJ*-specific (TRCN0000047606) shRNAs were either

696 used in the pLKO.1 vector or subcloned into the pLVX-shRNA2 vector (No. PT4052-5;
697 Clontech, Westburg BV, Leusden, the Netherlands). Scrambled shRNAs or the empty
698 vectors were used as negative controls (both with the same outcome). All constructs were
699 sequence verified. Lentiviral particles were produced in HEK293T cells as previously
700 described ².

701 **RECOMBINANT PROTEIN PRODUCTION:** Template vectors pRRLhGS, pRRLhGS^{R324C} and
702 pRRLhGS^{R341C} containing the gene encoding wild type or point mutated human GS were
703 used as templates for PCR-based cloning. Recombinant constructs were expressed in the
704 *Escherichia coli* strain BL21 codon + pICA2 that was transformed with pLH36-hGS in
705 which expression is induced by isopropyl b-D-1-thiogalactopyranoside under control of a
706 pL-promotor developed by the Protein Core of VIB (WO 98/48025, WO 04/074488). The
707 pLH36 plasmid is provided with a His₆-tag followed by a murine caspase-3 site. The
708 murine caspase-3 site can be used for the removal of the His₆-tag attached at the N-
709 terminus of the protein of interest during purification. The transformed bacteria were grown
710 in 200 ml Luria Bertani medium supplemented with ampicillin (100 µg ml⁻¹) and kanamycin
711 (50 µg ml⁻¹) overnight at 28 °C before 1/100 inoculation in a 20 l fermenter provided with
712 Luria Bertani medium supplemented with ampicillin (100 µg ml⁻¹) and 1 % glycerol. The
713 initial stirring and airflow was 200 rpm and 1.5 l min⁻¹, respectively. Further, this was
714 automatically adapted to keep the pO₂ at 30 %. The temperature was kept at 28 °C. The
715 cells were grown to an optical density of A_{600nm} = 1.0, transferred at 20 °C, and expression
716 was induced by addition of 1 mM isopropyl b-D-1-thiogalactopyranoside overnight. Cells
717 were then harvested and frozen at -20 °C. After thawing, the cells were resuspended at 3
718 ml g⁻¹ in 50 mM Hepes pH 7.5, 500 mM NaCl, 20mM imidazole, 1 mM phenyl-
719 methylsulfonyl fluoride, 10 % glycerol, 5 mM β-mercaptoethanol, 1 mg per 100 ml DNaseI
720 (Roche) and 1 tablet per 100 ml Complete Protease Inhibitor (Roche). The cytoplasmic
721 fraction was prepared by using the Emulsiflex followed by centrifugation. All steps were

722 conducted at 4 °C. The clear supernatant was applied to a 20 ml Ni-Sepharose 6 FF
723 column (GE Healthcare), equilibrated with 50 mM Hepes pH7.5, 500 mM NaCl, 20mM
724 imidazole, 10 % glycerol, 5 mM β -mercaptoethanol and 1 mM phenyl-methylsulfonyl
725 fluoride. The column was eluted with 50 mM Hepes pH 7.5, 500 mM NaCl, 400 mM
726 imidazole, 10 % glycerol, 5 mM β -mercaptoethanol and 1 mM phenyl-methylsulfonyl
727 fluoride after an intermediate elution step with 50 mM imidazole in the same buffer. Finally,
728 the elution fraction was injected on a HiLoad 26/60 Superdex prep grade with 20 mM
729 Hepes pH 7.5, 300 mM NaCL, 10 % glycerol and 0.5 mM TCEP as running solution. The
730 obtained elution fractions were analyzed by SDS-PAGE. Recombinant protein
731 concentration was determined using the Micro-BCA assay (Pierce).

732 ***IN VITRO* KNOCK-DOWN/OVEREXPRESSION STRATEGIES:** To minimize off-target effects and
733 other silencing artifacts, key findings were confirmed with at least two independent and
734 validated *GS*-specific shRNAs (see above) and appropriate controls or with a *GS*-specific
735 siRNA duplex (5'-GGAAUAGCAUGUCACUAAAGCAGGC-3') and scrambled control
736 (TriFECTaTM, IDT). For lentiviral transduction of shRNAs or overexpressing constructs an
737 MOI of 10 or 5 was used, respectively. In case of simultaneous transduction of 2 different
738 shRNAs, a MOI 7.5 was used for each individual shRNA. In case of simultaneous
739 transduction of a shRNA in combination with an overexpression construct, the shRNA was
740 transduced at MOI 10 and the overexpression construct at MOI 5, except for
741 overexpression constructs for shRNA-resistant *GS* which were transduced at MOI 2.5.
742 Transductions were performed on day 0 in the evening, cells were refed with fresh medium
743 on day 1 in the morning and experiments were performed from day 3 or 4 onwards. siRNA
744 transfection mixtures (in a total volume of 500 μ l) were prepared in Opti-MEM containing
745 GlutaMAX-I (Invitrogen) with Lipofectamine RNAi Max transfection reagent (Invitrogen,
746 Belgium) according to the manufacturer's instructions. The mixtures were added to the
747 cells (150,000 cells in 6 well-format plate) together with 2 ml EBM2 without antibiotics for

748 overnight transfection after which the medium was changed back to the regular M199
749 culture medium. siRNA transfection was done at least 48 h prior to functional assays. BiFC
750 plasmids were transfected into HEK293T cells with Fugene® HD transfection reagent
751 following the manufacturer's guidelines. KD efficiency and overexpression levels were
752 closely monitored for each experiment either on mRNA (QRT-PCR) or protein level.

753 **RNA ISOLATION AND GENE EXPRESSION ANALYSIS:** Total RNA was extracted with Invitrogen's
754 PureLink RNA mini kit according to the manufacturer's instructions; quality and quantity
755 were measured on a Nanodrop (Thermo Scientific). cDNA synthesis was performed with
756 the iScript cDNA synthesis kit (BioRad). Quantitative RT-PCR analyses were performed as
757 previously described ¹ on an Applied Biosystems 7500 Fast device with in house-designed
758 primers and probes or premade primer sets (Applied Biosystems or Integrated DNA
759 Technologies) for which sequences and/or primer set ID numbers are available upon
760 request. *ENOX2* or *HPRT* were used as housekeeping genes.

761 **WESTERN BLOTTING AND (CO-)IMMUNOPRECIPITATION:** Proteins were extracted in Laemmli
762 buffer (125 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol) or in RIPA buffer (25 mM Tris-
763 HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing
764 protease and phosphatase inhibitor mixes (Roche Applied Science). After shearing of
765 genomic DNA, proteins in the lysates were separated by SDS-PAGE, transferred to
766 nitrocellulose or polyvinylidene difluoride membranes and detected with specific antibodies
767 and HRP-conjugated secondary antibodies in combination with ECL or SuperSignal Femto
768 Western blotting substrate (Thermo Scientific). Signal was acquired with Image Quant LAS
769 4000 V 1.2 and densitometric quantification was done with ImageJ. For MLC and pMLC
770 immunoblotting, each sample was loaded on two separate gels. One gel was used to
771 detect MLC and the second was used to detect pMLC. Both gels had their own loading
772 control, namely α -tubulin. pMLC/MLC was quantified as follows: (pMLC/ α -tubulin)/(MLC/ α -

773 tubulin), abbreviated in the figure panel as (c)pMLC/(c)MLC with (c) meaning 'corrected for
774 corresponding loading control'. Membrane *versus* cytosolic protein fractions were purified
775 with the Plasma Membrane Protein Extraction Kit (101Bio) according to the manufacturer's
776 guidelines and using proprietary buffers. For co-immunoprecipitation (co-IP) of
777 endogenous or overexpressed proteins, ECs were lysed by rotating at 4 °C during at least
778 4 h in co-IP lysis buffer (20 mM Tris-HCl pH8, 137 mM NaCl, 10% glycerol, 1% nonidet
779 NP-40 and 2 mM EDTA). Equal amounts of protein were incubated overnight with specific
780 antibodies or matching isotype control IgGs at 4 °C. Subsequently, 20 µl of protein A/G-
781 Sepharose beads was added to the immune complexes for 4 h at 4 °C under gentle
782 rotation. The beads were pelleted, washed three times with ice-cold co-IP lysis buffer and
783 boiled for 5 min in reducing agent and loading buffer prior to SDS-PAGE. To determine the
784 impact of deleting RHOJ's first 20 N-terminal AAs on the interaction with GS, co-IPs were
785 done as above on ECs simultaneously overexpressing GS and RHOJ-EGFP or ΔN20-
786 RHOJ-EGFP. In some of the experiments the expression of the ΔN20-RHOJ-EGFP was
787 lower than the expression of RHOJ-EGFP. To correct for this possible bias, densitometric
788 quantification of all bands was performed in ImageJ and signals in the IP lanes were
789 normalized to the input signals. The amount of GS IP'ed was the same in the RHOJ-EGFP
790 and ΔN20-RHOJ-EGFP condition (data not shown).

791 **BIOCHEMICAL AND METABOLIC ASSAYS:** *BICINCHONINIC ACID (BCA) ASSAY* (Pierce) was used to
792 determine protein content with Gen5 1.11.5 (BioTek Instruments). *LDH RELEASE* as a
793 measure for cell survival was determined with the Cytotoxicity Detection Kit (Roche
794 Applied Science) with Gen5 1.11.5 (BioTek Instruments). *INTRACELLULAR REACTIVE OXYGEN*
795 *SPECIES (ROS) LEVELS* were determined by CM-H₂DCFDA dye (Invitrogen) labeling
796 following manufacturer's guidelines. *GLUTAMINE SYNTHETASE ACTIVITY* in living cells. The
797 enzyme activity in living cells was determined by pulse-labeling the cells for 30 min with 2
798 mM ¹⁵NH₄Cl and subsequent determination of ¹⁵N incorporation in intracellular glutamine

799 by gas chromatography - mass spectrometry GC-MS (see below). Similarly, GS activity
800 was measured by pulse-labeling for 30 min with 0.5 mM [U-¹³C]-glutamic acid and
801 subsequent tracing of ¹³C into glutamine by GC-MS. The 0.025 mM glutamine condition
802 was added to this assay for the sole purpose of having a positive control – lowering
803 external glutamine levels should increase GS activity – and are not in any way reflecting
804 maximal GS activity. Background signals were determined by pre-incubating the cells with
805 the GS inhibitor MSO. As an independent manner (not relying on labeling one of the
806 immediate substrates (NH₄⁺ or glutamate)) to determine GS activity, we performed steady
807 state labeling of ECs with [U-¹³C]-glucose (5.5 mM) and determined carbon contribution to
808 α-ketoglutarate, glutamate and glutamine (for labeling scheme see Extended Data Fig. 5f).
809 Prior to derivatization for GC-MS analysis, cells were washed with ice-cold 0.9% NaCl and
810 extracted in ice cold 80/20 methanol/water. *GLUTAMINE UPTAKE ASSAY*: Dynamic [U-¹³C]-
811 glutamine uptake assays were performed as follows: 2.5 x 10⁵ cells/well were seeded in 6
812 well plates and pulse-labeled for 0, 0.5, 10, 20 and 30 min with the regular M199 culture
813 medium containing 0.6 mM [U-¹³C]-glutamine instead of the regular 0.6 mM unlabeled
814 glutamine. The 0 min time point represents an absolute negative control for which extracts
815 were made from ECs that were never treated with tracer-containing medium. For the 0.5
816 min time point, the labeled medium was put on the cells and immediately aspirated (all
817 together taking 0.5 min). At all time points, cells were thoroughly washed twice with ice-
818 cold 0.9% NaCl to ensure complete removal of tracer-containing medium. Cellular extracts
819 were then made in ice-cold 80/20 methanol/water, prior to derivatization for GC-MS
820 measurements. Alternatively, cells were incubated with 0.5 μCi ml⁻¹ [U-¹⁴C]-L-glutamine for
821 10 min after which they were washed at least three times with ice-cold PBS. The last PBS
822 wash was collected and checked for residual radioactivity. Cells were then lysed with 200
823 μl 0.2 N NaOH and lysates were neutralized with 20 μl 1 N HCl and used for scintillation
824 counting. [³H]-*THYMIDINE INCORPORATION*: Proliferation was determined by labeling the cells

825 with $1 \mu\text{Ci ml}^{-1}$ [^3H]-thymidine for 2 h, followed by fixation in 100% ethanol for 15 min,
826 precipitation with 10% trichloroacetic acid and finally lysis in 0.1 N NaOH. Scintillation
827 counting was used to assess the amount of [^3H]-thymidine incorporated into the DNA.
828 *ENERGY CHARGE ASSESSMENT:* 1.5×10^6 cells were collected in 100 μl ice cold 0.4 M
829 perchloric acid containing 0.5 mM EDTA. pH was adjusted with 100 μl of 2 M K_2CO_3 . 100
830 μl of the mixture was subsequently injected onto an Agilent 1260 HPLC with a C18-
831 Symmetry column (150 x 4.6 mm; 5 mm; Waters), thermostated at 22.5 °C. Flow rate was
832 kept constant at 1 ml min^{-1} . A linear gradient using solvent A (50 mM NaH_2PO_4 , 4 mM
833 tetrabutylammonium, adjusted to pH 5.0 with H_2SO_4) and solvent B (50 mM NaH_2PO_4 , 4
834 mM tetrabutylammonium, 30% CH_3CN , adjusted to pH 5.0 with H_2SO_4) was accomplished
835 as follows: 95% A for 2 min, from 2 to 25 min linear increase to 100% B, from 25 to 27 min
836 isocratic at 100% B, from 27 to 29 min linear gradient to 95% A and finally from 29 to 35
837 min at 95% A. ATP, ADP and AMP were detected at 259 nm. *SEAHORSE EXTRACELLULAR*
838 *FLUX MEASUREMENTS:* ECs were seeded at 1.5×10^5 cells per well on Seahorse XF24
839 tissue culture plates (Seahorse Bioscience Europe). Oxygen consumption (OCR)
840 measurements were performed at 6 min intervals (2 min mixing, 2 min recovery, 2 min
841 measuring) in a Seahorse XF24 device (XF Reader 1.8.1.1 software). Consecutive
842 treatments with oligomycin (1.2 μM final), FCCP (5 μM final) and antimycin A (1 μM final)
843 were performed to allow quantification of ATP-coupled OCR (OCR_{ATP}) and maximal
844 respiration, next to basal OCR (OCR_{bas}). *GLYCOLYTIC FLUX:* ECs were cultured for 6 h in
845 medium containing 0.4 mCi ml^{-1} [$5\text{-}^3\text{H}$]-D-glucose (Perkin Elmer) after which supernatant
846 was transferred into glass vials sealed with rubber stoppers. $^3\text{H}_2\text{O}$ was captured in
847 hanging wells containing a Whatman paper soaked with H_2O over a period of 48 h at 37
848 °C to reach saturation ¹. Then the paper was used for liquid scintillation counting
849 (QuantaSmart TM V4 PerkinElmer). *^{14}C -GLUCOSE OXIDATION:* ECs were incubated for 6 h
850 in medium containing 0.55 mCi ml^{-1} [$6\text{-}^{14}\text{C}$]-D-glucose. After that, 250 μl of 2 M perchloric

851 acid was added to each well to stop cellular metabolism and to release $^{14}\text{CO}_2$, which was
852 captured overnight at room temperature in 1x hyamine hydroxide-saturated Whatman
853 paper. The radioactivity in the paper was determined by liquid scintillation counting
854 (QuantaSmart TM V4 PerkinElmer) ¹. *^{14}C -GLUTAMINE OXIDATION:* ECs were incubated for 6
855 h with medium containing 0.5 mCi ml⁻¹ [U- ^{14}C]-glutamine. 250 ml of 2 M perchloric acid
856 was added to the cells to stop cellular metabolism and release $^{14}\text{CO}_2$. Trapping of $^{14}\text{CO}_2$
857 occurred as described above for glucose oxidation ¹.

858 **PROTEIN (AUTO)PALMITOYLATION DETECTION:** *IN VITRO PALMITOYLATION (CLICK REACTION-*
859 *BASED):* Purified bacterial GS protein was incubated with the indicated concentration of
860 palmitoyl alkyne-coenzyme A (Cayman Chemical) for 6 h at room temperature. The GS
861 protein was then denatured by the addition of SDS. A click reaction with azide-biotin was
862 performed to label the palmitoylated proteins ²⁵. Palmitoylated proteins were detected by
863 SDS-PAGE followed by blotting with streptavidin-horseradish peroxidase. *FLUORESCENCE-*
864 *BASED COA RELEASE DETECTION:* During autopalmitoylation of proteins, palmitate is
865 transferred from palmitoyl-CoA to the protein thereby releasing reduced CoA. α -
866 Ketoglutarate dehydrogenase can use CoA to convert α -ketoglutarate to succinyl-CoA, a
867 reaction that features reduction of NAD^+ to fluorescent NADH^{29} . In brief, recombinant
868 human GS was incubated with palmitoyl-CoA in MES buffer at physiological pH for at least
869 1 h at 30 °C. The volume was then adjusted to 200 μl in 50 mM sodium phosphate buffer
870 (pH 6.8) containing 2 mM α -ketoglutaric acid, 0.25 mM NAD^+ , 0.2 mM thiamine
871 pyrophosphate, 1 mM EDTA, 1 mM DTT and 32 mU α -ketoglutarate dehydrogenase.
872 NADH levels were measured at 20 min after initiation of the reaction on a VICTOR plate
873 reader (340 nm excitation – 465 nm emission). The experiment was performed in two
874 directions: either with varying doses of palmitoyl-CoA for a fixed amount of recombinant
875 GS or with varying amounts of recombinant GS for a fixed concentration of palmitoyl-CoA
876 (40 μM). *AFFINITY CHROMATOGRAPHY:* A previously published protocol was used to

877 determine cell-free binding of recombinant human GS to palmitoyl-CoA agarose³⁰. A total
878 of 50 μ l of immobilized palmitoyl-CoA-agarose was equilibrated with 20 mM Tris-HCl (pH
879 8.4)/120 mM NaCl. The beads were incubated with 40 μ g of recombinant human GS in a
880 final volume of 200 μ l for 2 h at room temperature on a rotatory system. Beads were
881 pelleted and 20 μ l of the supernatant was collected as the flow through (FT) fraction.
882 Beads were then washed eight times with 500 μ l of 20 mM Tris-HCl (pH 8.4)/120 mM NaCl
883 buffer. 20 μ l of the last wash fraction was collected as fraction W8. Beads were then eluted
884 with SDS loading buffer and heated for 15 min at 60 °C. 2 μ g of recombinant protein was
885 used as input fraction (IF). IF, FT, W8 and SDS-eluate were analysed by immunoblotting
886 for GS. *IN CELL LABELING*: In cell labeling experiments were performed essentially as
887 described previously²⁵. HEK-293T cells were transfected with the indicated expression
888 plasmids. Twenty-four h after transfection, the medium was replaced with DMEM + 10%
889 dialyzed FBS containing the indicated probes (50 μ M 16C-YA or 50 μ M 16C-BYA). After
890 18 h, cell lysates were collected by incubation of the cells on ice for 15 min in lysis buffer
891 (50 mM TEA-HCl (pH=7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate,
892 0.1% SDS and 5 mM PMSF) followed by centrifugation for 10 min at 15,000 *g*. Equal
893 amounts of protein were then used for a click reaction with azide-biotin. For labeling with
894 17-ODYA, FLAG-RHOJ overexpressing ECs were incubated overnight with 17-ODYA (50
895 μ M) in M199 supplemented with 3.6% fatty acid free BSA, 10% dialyzed FBS and 5 mM
896 sodium pyruvate. Cells were washed with ice-cold PBS and lysed in NaP lysis buffer (0.2
897 M Na₂HPO₄·2H₂O, 0.2 M NaH₂PO₄·2H₂O, 1 M NaCl, 10% NP40). 2 μ g of anti-Flag
898 antibody was conjugated to 20 μ l of dynabeads protein G (ThermoFisher) for 1 h at RT.
899 After washing the beads twice with NaP lysis buffer, at least 500 μ g of protein was added
900 to the beads for 3 h at 4 °C. Then beads were washed 3 times with NaP lysis buffer and
901 resuspended in 20 μ l of resuspension buffer (4% SDS, 50 mM TEA, 150 mM NaCl). The
902 click reaction was initiated by adding 0.5 μ l of 5 mM tetramethylrhodamine azide (TAMRA)

903 (Lumiprobe), 0.5 μ l 50 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), 0.5 μ l
904 10 mM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) and 2.4 μ l of 5 mM freshly
905 made ascorbic acid. Samples were then incubated for 1 h at 37 °C in the dark. Sample
906 buffer (9.4 μ l) and reducing agent (3.7 μ l) were added to stop the reaction. After 10 min at
907 room temperature in the dark, samples were frozen at -80 °C or run on a 10% Bis-TRIS
908 gel in MES buffer. In-gel fluorescence was imaged with Typhoon TM FLA 9500 V1.0.
909 *STREPTAVIDIN-PULLDOWN*: After click reaction with azide-biotin, free azide-biotin was
910 removed from the samples by centrifugal filtration column (Millipore). The samples were
911 then incubated with streptavidin-conjugated beads for 1 h at room temperature. After
912 washing with PBS-T, proteins were eluted from the beads by incubation in elution buffer
913 (95% formamide, 10 mM EDTA (pH=8.0)) at 95 °C for 5 min. *ACYL-RESIN-ASSISTED*
914 *CAPTURE (ACYL-RAC)* in which free cysteine thiols are chemically blocked and palmitoylated
915 cysteines are exposed and captured by a resin, was performed with the CAPTUREome™
916 S-Palmitoylated Protein Kit (Badrilla) with minor adaptations to the manufacturer's
917 guidelines. 500 μ g of protein were incubated for 4 h in 500 μ l of thiol blocking reagent (to
918 block free thiols). Proteins were precipitated with ice-cold acetone and afterwards
919 solubilized with 300 μ l of binding buffer and spun down. After protein quantification, 30 μ g
920 was kept as total input fraction (IF), and equal amounts of protein were incubated for 2.5 h
921 with (or without to obtain the negative control preserved bound fraction (pBF)) a thioester
922 linkage specific cleavage reagent to cleave the thioester bond. Newly liberated thiols were
923 captured with CAPTUREome™ resin. The resin was spun down and the supernate was
924 collected as the cleaved unbound fraction (cUF) to check if the proteins of interest were
925 indeed completely depleted from the thioester cleavage reagent (meaning efficient capture
926 of the free thiols by the resin). After thorough washing of the resin, captured proteins
927 (cleaved bound fraction (cBF)), were eluted with reductant and analyzed together with the
928 IF, cUF and pBF by SDS-PAGE followed by immunoblotting.

929

930 **GC-MS ANALYSIS:** Metabolites from cells were extracted in 800 μl 80% methanol (at -80
931 $^{\circ}\text{C}$). Next the extracts were centrifuged at 4 $^{\circ}\text{C}$ for 15 min at 20,000 x g and the
932 supernatants were dried in a vacuum centrifuge. 25 μl of a 2% methoxyamine
933 hydrochloride solution (20 mg dissolved in 1 ml pyridine) was added to the dried fractions
934 which were then incubated at 37 $^{\circ}\text{C}$ for 90 min. Then 75 μl of N-tert-butyldimethylsilyl-N-
935 methyltrifluoroacetamide with 1% N-tert-butyldimethyl-chlorosilane (Sigma-Aldrich) was
936 added and the reaction was carried out for 30 min at 60 $^{\circ}\text{C}$. Reaction mixtures were
937 centrifuged for 15 min at 20,000 x g at 4 $^{\circ}\text{C}$ in order to remove insolubilities and the
938 supernatant was transferred to a glass vial with conical insert (Agilent). GC-MS analyses
939 were performed on an Agilent 7890A GC equipped with a HP-5 ms 5% Phenyl Methyl
940 Silox (30 m - 0.25 mm i.d. - 0.25 μm ; Agilent Technologies) capillary column, interfaced
941 with a triple quadrupole tandem mass spectrometer (Agilent 7000B, Agilent Technologies)
942 operating under ionization by electron impact at 70 eV. The injection port, interface and ion
943 source temperatures were kept at 230 $^{\circ}\text{C}$. Temperature of the quadrupoles was kept at
944 150 $^{\circ}\text{C}$. The injection volume was 1 μl , and samples were injected at 1:10 split ratio.
945 Helium flow was kept constant at 1 ml min^{-1} . The temperature of the column started at 100
946 $^{\circ}\text{C}$ for 5 min and increased to 260 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C min}^{-1}$. Next, a 40 $^{\circ}\text{C min}^{-1}$ gradient was
947 carried out until the temperature reached 300 $^{\circ}\text{C}$. After the gradient, the column was
948 heated for another 3 min at 325 $^{\circ}\text{C}$. The GC-MS analyses were performed in Single Ion
949 Monitoring (SIM) scanning for the isotopic pattern of metabolites.

950 **LC-MS ANALYSIS:** *POLAR METABOLITES* were extracted using 250 μl of a 50-30-20
951 (methanol-acetonitrile-10 mM ammonium acetate pH 9.3 containing 2 μM of deuterated
952 (d27) myristic acid as internal standard) extraction buffer. Following extraction, precipitated
953 proteins and insolubilities were removed by centrifugation at 20,000 x g for 20 min at 4 $^{\circ}\text{C}$.
954 The supernatant was transferred to the appropriate mass spectrometer vials.

955 Measurements were performed using a Dionex UltiMate 3000 LC System (Thermo
956 Scientific) in-line connected to a Q-Exactive Orbitrap mass spectrometer (Thermo
957 Scientific). 15 μl of sample was injected and loaded onto a Hilicon iHILIC-Fusion(P)
958 column (Achrom). A linear gradient was carried out starting with 90% solvent A (LC-MS
959 grade acetonitrile) and 10% solvent B (10 mM ammonium acetate pH 9.3). From 2 to 20
960 mins the gradient changed to 80% B and was kept at 80% until 23 min. Next a decrease to
961 40% B was carried out to 25 min, further decreasing to 10% B at 27 min. Finally, 10% B
962 was maintained until 35 min. The solvent was used at a flow rate of $200 \mu\text{l min}^{-1}$, the
963 column's temperature was kept constant at $25 \text{ }^\circ\text{C}$. The mass spectrometer operated in
964 negative ion mode, settings of the HESI probe were as follows: sheath gas flow rate at 35,
965 auxiliary gas flow rate at 10 (at a temperature of $260 \text{ }^\circ\text{C}$). Spray voltage was set at 4.8 kV,
966 temperature of the capillary at $300 \text{ }^\circ\text{C}$ and S-lens RF level at 50. A full scan (resolution of
967 140,000 and scan range of m/z 70-1050) was applied. For the data analysis, we used an
968 in-house library and metabolites of interest were quantified (area under the curve) using
969 the XCalibur 4.0 (Thermo Scientific) software platform.

970 **IN VITRO ASSAYS:** *ENDOTHELIAL SPHEROID CAPILLARY SPROUTING* was performed following
971 established protocols ^{1,2}. To form the spheroids, ECs were cultured overnight in hanging
972 drops in EGM2 medium with methylcellulose (Sigma-Aldrich; 20 %volume of a 1.2%
973 solution of methylcellulose 4000 cP). Spheroid sprouting entails both EC proliferation and
974 migration. To have a 'clean' view on the migration aspect in sprouting, we also included
975 conditions in which we blocked EC proliferation prior to sprout formation. More in
976 particular, mitotic inactivation was achieved by adding mitomycin C ($1 \mu\text{g ml}^{-1}$) to the
977 medium. To induce sprouting, spheroids were embedded in a collagen gel and incubated
978 for 20 h. If required, chemical compounds (Fasudil at $10 \mu\text{M}$, H1152 at $1 \mu\text{M}$ and Y26732
979 at $10 \mu\text{M}$) were added during the collagen gel incubation step. Spheroids were then fixed
980 with 4% paraformaldehyde and imaged under phase contrast illumination with a Motic AE

981 31 microscope (Motic Electric Group Co Ltd) or a Leica DMI6000B microscope (Leica
982 Microsystems). Phase contrast images were used to quantify the number of sprouts per
983 spheroid and the total sprout length (cumulative length of all sprouts on a spheroid).
984 Spheroid body circumference was measured to correct for differences in size of the
985 spheroid. Per experiment (*ie* per individual HUVEC isolation) at least 10 spheroids per
986 condition were analyzed. *SCRATCH WOUND ASSAYS*: 75,000 HUVECs were seeded in 24-
987 well format and were allowed to reach confluency over the next 24 h. At time T0 the
988 confluent monolayer was scratched with a 200 μ l pipet tip and photographed. The cells
989 were further incubated for the indicated times and photographed again at time point Tx.
990 Gap area at T0 minus gap area at Tx was measured with ImageJ and expressed as %
991 migration distance. Per well, three non-overlapping regions along the scratch were
992 analyzed. Much like the spheroid sprouting, scratch wound healing is a combined readout
993 for EC migration and proliferation. Therefore, we also included conditions in which the ECs
994 were pre-treated with mitomycin C ($1 \mu\text{g ml}^{-1}$) to rule out the effect of proliferation. *BOYDEN*
995 *CHAMBER ASSAYS*: 50,000 HUVECs were seeded on 0.1% gelatin-coated transwells and
996 allowed to adhere. Then, the transwells were washed and re-fed with medium containing
997 only 0.1% FBS and placed in bottom wells containing medium with 5% FBS as a pro-
998 migratory stimulus. 16 h later, transwells were processed and analysed for numbers of
999 migrated cells. Pre-treatment with mitomycin C (see above) was applied. *VELOCITY OF*
1000 *RANDOM MOVEMENT* was assessed on HUVECs that were sparsely seeded on glass bottom
1001 24-well plates. Time-lapse videos were generated by confocal image acquisition at 4 min
1002 intervals. Velocity of movement was determined by tracking nucleus position in function of
1003 time ($\mu\text{m h}^{-1}$) (Tracking Tool TM, Gradientech AB, Uppsala, Sweden). Per condition, on
1004 average 2 or 3 individual cells were traced in each biological repeat. *LAMELLIPODIAL AREA*
1005 was measured on sparsely seeded phalloidin-stained ECs with Leica MM AF
1006 morphometric analysis software (Leica Microsystems, Mannheim, Germany) with in-house

1007 developed journals and is expressed in percent of total cell area. Treatment with MSO (1
1008 mM), Y27632 (10 μ M), Fasudil (10 μ M), H1152 (1 μ M), ML7 (15 μ M) and peptide 18 (15
1009 μ M) were done 24 h prior to analysis of the cells. Per experimental condition, a minimum
1010 of ten individual cells was analyzed. *STAINING AND QUANTIFICATION OF VE-CADHERIN*
1011 *JUNCTIONS*: VE-cadherin staining and quantification of junctional length and gap index was
1012 performed as previously described ³¹. First, the total junctional length (100%) was
1013 determined by summing up all segments, then the sum of all continuous segments was
1014 calculated as the percentage of total junctional length. The percentage difference between
1015 total and continuous represents the discontinuous length. Gap size index (intercellular gap
1016 area/cell number) was determined with the formula ($[\text{intercellular gap area}/\text{total cell area}]$
1017 $\times 1,000$)/cell number. Junctional lengths, intercellular gap area, and total cell area were
1018 defined manually with ImageJ. For each condition, a minimum of 10 fields was quantified
1019 (10-15 cells per field on average) per experiment, and data shown represent the mean of
1020 at least 3 independent experiments. *TRANS ENDOTHELIAL ELECTRICAL RESISTANCE (TEER)*:
1021 50,000 ECs were seeded on 6.5 mm 0.1% gelatin-coated polyester transwells, 0.4 μ m
1022 pore size (Costar ref. 3470, Sigma-Aldrich). The electrical resistance was measured with
1023 an Endhome-6 electrode (World Precisions Instruments) connected to an EVOM2
1024 voltohmmeter (World Precisions Instruments). Gelatin-coated wells without cells were
1025 used to measure the intrinsic electrical resistance of the inserts for background
1026 subtraction. Measurements were performed every day for 4 consecutive days, with at least
1027 2 measurements per condition.

1028 **ACTIN DYNAMICS AND RHO (KINASE) ACTIVITY ASSAYS: LATRUNCULIN WASH-OUT**. ECs were
1029 treated with latrunculin B (100 ng ml⁻¹) for 30 min and were then washed three times with
1030 culture medium. The cells were fixed at the indicated time points and stained with
1031 phalloidin to visualize actin stress fibers. *THE F-/G-ACTIN RATIO* in GS^{KD} vs control ECs was
1032 determined in 4% paraformaldehyde-fixed cells which were permeabilized for 10 min in

1033 PBS with 0.2% Triton X-100 and stained with phalloidin-Alexa 488 and deoxyribonuclease
1034 I-Alexa 594 (1:200)³². Fluorescence intensities were quantified with ImageJ and were
1035 based on gray values. On average, ten individual cells were analyzed per experimental
1036 condition. For *RHOJ ACTIVITY* measurements, cells were lysed in buffer containing 50 mM
1037 Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl₂, protease inhibitors and 0.1
1038 μg μl⁻¹ biotinylated CRIB-peptide. After spinning down for 4 min at 14,000 rpm at 4 °C, 50
1039 μl streptavidin-coated beads were added to the lysates. Subsequently, samples were
1040 rotated for 30 min at 4 °C, beads were washed 4 times in the above buffer after which they
1041 were boiled for 5 min in reducing agent and loading buffer³³. As negative controls in this
1042 assay, we used lysates from RHOJ^{KD} ECs, a streptavidin beads only-condition and lysates
1043 in which the biotinylated CRIB-peptide was replaced by an irrelevant biotinylated protein
1044 (Fig. 4c). *RHOA/B/C ACTIVITY* was determined with GST-Rhotekin pull down assays
1045 following previously established protocols³⁴. *ROCK ACTIVITY* was assayed by determining
1046 phosphorylation of the ROCK target myosin light chain 2 (MLC2) on Western Blot or by
1047 immunostaining. Fluorescence intensities from immunostainings were quantified with
1048 ImageJ and were based on gray values.

1049 **CONFOCAL AND HIGH RESOLUTION IMAGING:** *CONFOCAL IMAGING* was performed on a Zeiss
1050 LSM 510 Meta NLO or Zeiss LSM 780 confocal microscope (oil objectives: x 40 with NA
1051 1.3, x 63 with NA 1.4, x 100 with NA 1.3) with ZEN 2011 software (Carl Zeiss, Munich,
1052 Germany). Within individual experiments, all images across different experimental
1053 conditions were acquired with the same settings. *DORA RHOA BIOSENSOR FRET IMAGING:*
1054 RHOA activity was measured in living HUVECs by monitoring yellow fluorescent protein
1055 (YFP) FRET over donor cyan fluorescent protein (CFP) intensities as described previously
1056³⁵. In brief, a Zeiss Observer Z1 microscope, with a Chroma 510 DCSP dichroic splitter,
1057 two Hamamatsu ORCA-R2 digital CCD cameras and an attached dual camera adaptor
1058 (Zeiss) controlling a 510 DCSP dichroic mirror, was used for simultaneous monitoring of

1059 CFP and YFP emissions using filter sets ET 480/40 and ET 540/40m (Chroma
1060 Technology, Rockingham, USA), respectively. To excite the CFP donor, ET 436/20x and
1061 455 DCLP dichroic mirror was used (Chroma). For FRET/CFP ratiometric processing, CFP
1062 and YFP images were processed using the MBF ImageJ collection. The images were
1063 background-subtracted, aligned and a threshold was applied. Finally, the FRET/CFP ratio
1064 was calculated and a custom lookup table was applied to generate a color-coded image, in
1065 which white and red colors illustrate high and blue colors illustrate low RHOA activities.

1066 *BiFC IMAGING AND QUANTIFICATION:* BiFC was evaluated using a laser scanning microscope
1067 (Fluoview FV1000, Olympus, Tokyo, Japan) equipped with a UPLSAPO 60x Oil objective
1068 (NA1.35). Before imaging cells were fixed with 4 % (v/v) paraformaldehyde and stained
1069 with DAPI (1:1,000 dilution, Invitrogen). A 488-nm laser was used for exciting EGFP while
1070 DAPI was excited using a 405-nm laser. A DM405/488/559/635 polychroic mirror was
1071 used to guide the excitation lasers to the sample. Fluorescence images of fixed cells were
1072 acquired using a sampling speed of 4 $\mu\text{s pixel}^{-1}$. Emission light was collected at 430-470
1073 and 500-550 nm, for DAPI and EGFP, respectively. The images were acquired with a pixel
1074 size of 207 nm (1,024 x 1,024 pixels). BiFC was first established in HEK cells expressing
1075 GS-EGFP^{1/2} and RHOJ-EGFP^{2/2} from one expression vector, with a construct
1076 overexpressing an unfused N-terminal EGFP half-site together with RHOJ coupled to the
1077 C-terminal EGFP half-site as a negative control (data not shown). To determine the effect
1078 of deleting the first 20 amino acids in RHOJ on BiFC in ECs, separate expression
1079 constructs for GS-EGFP^{1/2}, RHOJ-EGFP^{2/2} and $\Delta\text{N-RHOJ-EGFP}^{2/2}$ were used (Ext. Data
1080 Fig. 7e). Quantification of expression efficiency was done using a home-built routine in
1081 Matlab®. *TIRF MICROSCOPY:* A home build setup based on an inverted microscope (IX83,
1082 Olympus) was used to detect single molecules under total internal reflection (TIRF) mode.
1083 The setup was equipped with an Electron Multiplying-CCD cameras (ImagEM C9100-13;
1084 Hamamatsu Photonics, Hamamatsu, Japan) and an APON 60XOTIRF objective lens (NA

1085 1.49, Olympus). The GS-mEos3.2 molecules were excited with a 561-nm line from a
1086 DPSS laser (200 mW; Coherent Inc., Santa Clara, California) and converted with a 405-
1087 nm line from a diode laser (Cube, 100 mW; Coherent Inc., Santa Clara, California). Before
1088 being expanded, the laser lines were combined using a 405bcm dichroic mirror. The laser
1089 lines were guided onto the sample by a dichroic mirror, z488/561/633rpc. The
1090 fluorescence of the red of mEos3.2 form was detected through a long pass filter 572
1091 (HQ572LP), in combination with a band pass filter HQ590M40-2P. All the filters were
1092 purchased from Chroma Inc. Time-lapse fluorescence images were recorded with
1093 continuous illumination at a 62.5 Hz acquisition rate (16 ms per frame). *SINGLE PARTICLE*
1094 *TRACKING (SPT)*: For calculation of single molecule coordinates the program 'Localizer'
1095 running from Matlab was used ³⁶. After localization, the positions of a molecule detected in
1096 consecutive frames are connected to reconstruct a trajectory using home-developed
1097 software in Matlab. Coordinates presented in consecutive frames are linked to form a
1098 single trajectory when they uniquely appear in a distance smaller than 856 nm
1099 (corresponding to 8 pixels). Trajectories with at least 3 steps were analyzed using
1100 variational Bayes single particle tracking analysis (vbSPT), a software package for
1101 analysis of single particle diffusion trajectories, where the diffusion constants switch
1102 randomly according to a Markov process ³⁷.

1103 **MICE:** GS^{ECKO} MICE: To obtain inducible EC-specific GS knock-out mice, $GS^{lox/lox}$ mice ³⁸
1104 were intercrossed with $VECadherin-Cre^{ERT2}$ ³⁹ or with $Pdgfb-Cre^{ERT2}$ ⁴⁰ mice and named
1105 GS^{vECKO} and GS^{pECKO} respectively. Correct Cre-mediated excision of the loxed GS
1106 segment in tamoxifen-treated GS^{ECKO} mice was confirmed via PCR analysis of genomic
1107 DNA (Extended Data Fig. 1d-e). *GENERATION OF $GS^{+/GFP}$ CHIMERAS* : Blastocysts were
1108 collected from superovulated C57BL/6 females at post-coital day 3.5 and were cultured for
1109 5-8 days in ES cell culture medium consisting of Knockout DMEM medium (Invitrogen),
1110 with 2 mM L-glutamine, fetal bovine serum (Hyclone, ThermoScientific), MEM non-

1111 essential amino acids 100X (Invitrogen), 0.01 mM β -mercaptoethanol (Sigma-Aldrich), 1
1112 mM sodium pyruvate (Invitrogen), 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, and
1113 2,000 U ml⁻¹ Leukemia Inhibitory Factor (Merck, Millipore). Afterwards, the inner cell mass
1114 was selectively removed from the trophectoderm, trypsinized and replated on a Mitomycin
1115 C-arrested MEF feeder monolayer. ES cells were fed every day and passaged every 2-4
1116 days onto new feeder cells. $GS^{+/GFP}$ ES cells (E14IB10 ES cell line)⁶ were injected into
1117 C57BL/6 blastocysts and high chimeric pups were killed at P5 for detection of GFP in the
1118 retinal microvasculature.

1119 ***IN VIVO MODELS: ANALYSIS OF DORSAL DERMAL BLOOD VESSEL NETWORK:*** From E11.5 to E13.5
1120 after vaginal plug, GS^{vECKO} pregnant dams were treated with tamoxifen (50 mg kg⁻¹) by
1121 oral gavage. At E16.5 they were euthanized by cervical dislocation after which embryos
1122 were dissected from the uterus. Yolk sacs were collected, washed with PBS and used for
1123 genotyping of the embryos. The embryos were fixed for 10 min in 1% PFA prior to
1124 dissection of the dorsal skin. The epidermal and dermal layers were separated under a
1125 dissection microscope. Dissected back skins were permeabilized overnight (0.5% Triton X-
1126 100, 0.01% sodium deoxycholate, 1% bovine serum albumin, 0.02% sodium azide) prior to
1127 whole-mount immunostaining with CD31. To systematically analyze the same region for
1128 each embryo, 1 rectangular confocal image (1,700 x 1,100 μ m) was taken at the anterior
1129 side of the skin specimen with the upper longer side of the rectangle placed on the
1130 midline. Within each rectangular picture the number of branch points was determined with
1131 the cell counter tool in ImageJ in 6 ROIs (250 x 250 μ m), 3 in the top half and 3 in the
1132 bottom half of the rectangle, not overlapping with the larger arteries and veins. ***NEONATAL***
1133 ***RETINAL ANGIOGENESIS:*** EC-specific GS deletion was obtained by IP administration of
1134 tamoxifen (Sigma; 10 mg kg⁻¹; dissolved in 1:10 EtOH:oil solution) once daily from P1 to
1135 P3 in GS^{vECKO} or once at P2 for GS^{pECKO} . For *in vivo* proliferation quantification, EdU (5-
1136 ethynyl-2'-deoxyuridine; Invitrogen) was injected IP 2 h before sacrifice. Unless stated

1137 otherwise, retinas were isolated at P5 as previously described⁴¹ and fixed in 2% PFA for 2
1138 h. Isolectin B4 (IB4), EdU, NG2 and CollIV stainings were performed as previously
1139 described^{1,2}. Radial outgrowth of the vascular plexus, vascular area, branch points,
1140 number of filopodia and number of distal sprouts were analysed on isolectin IB4-stained
1141 retinas (see below) with Image J. Numbers of branch points and EdU⁺ ECs were quantified
1142 in 200 x 200 μm ROIs; per retina 12 ROIs were placed at the front of the vascular plexus
1143 and 8 ROIs were placed more towards the center of the plexus. Filopodia and distal
1144 sprouts were quantified on ten high magnification (63x) images per retina, each
1145 representing approximately 200 μm of utmost vascular front. For analysis of the retinal
1146 vasculature at P21 (3 week-old) and P42 (6 week-old) mice underwent the same
1147 tamoxifen treatment regimen as for analyses at P5. In addition, different tissues were
1148 collected from P42 mice for endoglin and CD34 staining to study blood vessels in different
1149 vascular beds. *OXYGEN INDUCED RETINOPATHY*: Oxygen induced retinopathy (ROP) was
1150 induced by exposing C57BL/6 pups to 70% oxygen from P7-P12. Pups were then returned
1151 to normoxia and injected daily with 20 mg kg⁻¹ MSO. At P17, pups were euthanized and
1152 eyes were enucleated, fixed in 4% PFA and retinal flatmounts were stained for isolectin B4
1153^{2,3}. MSO-treated animals retained normal behavior notwithstanding observable weight
1154 loss. Mosaic tile images were captured using the inverted Leica DMI6000B
1155 epifluorescence microscope (Leica, Mannheim, Germany) and analysis of the vascular tuft
1156 area (the complete retina was analyzed, no ROIs were used) and the vaso-obiterated
1157 area was performed with NIH Image J software and are expressed as percentage of the
1158 total retinal area. *CORNEAL (MICRO-)POCKET ASSAY (CPA)* to induce neovascularization of
1159 the avascular cornea was performed as previously described⁴². In brief, in the eyes of 8
1160 week-old C57BL/6 mice, a lamellar micropocket was dissected toward the temporal limbus
1161 to allow placing of a basic fibroblast growth factor (bFGF)-containing pellet on the corneal
1162 surface. Five days after implanting the pellets, the mice were sacrificed, the eyes were

1163 enucleated and the corneas were excised and fixed in 70% ethanol prior to CD31 antibody
1164 staining. After staining, the corneas were flat-mounted and imaged on a Zeiss LSM 780
1165 confocal microscope. CD31⁺ area was measured in ImageJ after thresholding the signal
1166 and is expressed as % of total cornea area. Production of the pellets was done as
1167 previously described ⁴². The pellets contained 20 ng bFGF and the concentration of MSO
1168 in the initial solution from which the pellets were made was 10 mM. *IMIQUIMOD-INDUCED*
1169 *SKIN INFLAMMATION*: Ten week old female Balb/C mice received a daily topical dose of 5%
1170 imiquimod cream (62.5 mg) on their shaved backs for four days to induce skin
1171 inflammation ³. 1 h after each administration of the cream, the same skin area was treated
1172 either with Vaseline[®] jelly or Vaseline[®] jelly containing MSO (low dose: 20 mg kg⁻¹; or high
1173 dose: 40 mg kg⁻¹). The MSO treatment did not affect bodyweight of the mice. Skins and
1174 spleens were collected and fixed in 4% PFA. Paraffin sections of skins were stained for
1175 CD105 (R&D Systems) and H&E. Images were captured with a Leica DMI6000B
1176 microscope (Leica microsystems, Mannheim, Germany). Per animal, ten images
1177 representing different locations along the total length of the skin specimen were analyzed
1178 for CD105⁺ area. *MILES VASCULAR PERMEABILITY ASSAY*: 8 week old female Balb/C mice
1179 were treated for 3 consecutive days with 20 mg kg⁻¹ day⁻¹ MSO or with vehicle prior to
1180 injection with 300 µl 0.5 % Evan's blue dye. The inflammatory irritant mustard oil (0.25 ml
1181 allyl isothiocyanate in 4.75 ml mineral oil) was applied on one of the ears with a cotton
1182 swab to induce vascular permeability. Mineral oil as a control was applied on the other ear.
1183 After 15 min, again mustard oil/mineral oil was applied on the ear for 30 min, after which
1184 the circulation was flushed with saline for 3 min and mice were perfused with 1 % PFA in
1185 50 mM citrate buffer (pH=3.5) for 2 min. Ears were cut and minced in formamide and
1186 incubated at 55 °C overnight to extract the Evan's blue from the tissue. Quantification of
1187 the dye was performed by a spectrophotometrical optical density measurement at 620 nm.
1188 *HEMATOLOGICAL PROFILING IN 6 WEEK-OLD MICE* was performed with a Cell Dyn 3700 device

1189 (Abbott Diagnostics) according to the manufacturer's guidelines. Plasma measurements
1190 for different liver/inflammation parameters were performed in the clinical laboratory of the
1191 university hospital of Leuven. Prior randomization was not applicable for any of the above
1192 mouse models given that all animal treatments were done in baseline conditions. No
1193 statistical methods were used to predetermine the sample size. For all mouse
1194 experiments, data analysis was done by researchers blinded to the group allocation. All
1195 animal procedures were approved by the Institutional Animal Care and Research Advisory
1196 Committee of the University of Leuven.

1197 **IN SILICO SCREENING FOR PALMITOYLATION SITES:** The human RHOJ protein sequence was
1198 screened for putative palmitoylation sites on the SwissPalm website²² entering 'RHOJ' as
1199 the protein name.

1200 **MODELING AND SIMULATIONS:** The GS models were built starting from X-ray crystallographic
1201 structures retrieved from the Protein Data Bank (entry 2OJW for human GS and 1FPY for
1202 bacterial GS). All simulations were run with Gromacs 5.1.4⁴³ and the Amber FF14SB⁴⁴
1203 force field, while palmitoyl-CoA was parametrized with GAFF and the point charges were
1204 calculated with Gaussian 09⁴⁵ at the Hartree-Fock level with a 6-31G* basis set. The
1205 different models were then embedded in a TIP3P water box, counter ions were added to
1206 ensure the overall charge neutrality. An initial 2,000 steps of steepest descent and 500
1207 steps of conjugated gradient were applied to minimize the geometry and remove steric
1208 clashes, followed by 10 ns of isothermal-isobaric (NPT) equilibration. The Berendsen
1209 barostat was applied to keep the pressure around 1 atm, while the temperature of 300K
1210 was maintained throughout all the simulations with the V-rescale algorithm⁴⁶. 500 ns long
1211 molecular dynamics production runs were carried out for all the systems in the canonical
1212 (NVT) ensemble, for a cumulative total of 2.5 μ s. The particle mesh Ewald (PME)-Switch
1213 algorithm was used for electrostatic interactions with a cut-off of 1 nm, and a single cut-off

1214 of 1.2 nm was used for Van der Waals interactions. Four simulations for human GS and
1215 two for *Salmonella typhimurium*'s GS were run by placing the CoA moiety close to the
1216 adenosine binding site and allowing different initial positions for the palmitoyl tail. The CoA
1217 head invariably docked and remained tightly bound to the adenine binding site in all
1218 simulations. Among these, two favorable alternative arrangements (Extended Data Fig.
1219 8b) for the tail were identified in both systems. In one of these conformations, the
1220 beginning of the palmitate tail (from the point of view of the CoA moiety) approaches very
1221 closely the conserved CYS209 (human residue numbering, Conformation A in Extended
1222 Data Fig. 8b, details in Extended Data Fig. 8c), and in the other conformation
1223 (Conformation B in Extended Data Fig. 8b, details in Extended Data Fig. 8d) it approaches
1224 the conserved Ser65 and 75.

1225 **MULTIPLE SEQUENCE ALIGNMENTS:** A multiple sequence alignment of the GS protein across
1226 different species was performed with the Basic Local Alignment Search Tool (BLAST). The
1227 algorithm matches sequences according to local similarity, by optimizing their Maximal
1228 Segment Pair score (MSP). The 100 matches with the highest identity to the *Homo*
1229 *sapiens* sequence surrounding amino acid C209 were taken from the UniProtKB/Swiss-
1230 Prot refined database.

1231 **STATISTICAL ANALYSIS:** Data represent mean \pm s.e.m. of pooled experiments unless
1232 otherwise stated. Scatters in bar graphs represent the values of independent experiments
1233 or individual mice. In case individual values are highly alike, scatter points overlap and
1234 may no longer be visible as individual points. n values represent the number of
1235 independent experiments performed or the number of individual mice phenotyped.
1236 Statistical significance between groups was calculated with one of the following methods.
1237 For comparisons to point-normalized data, a two-tailed one-sample *t*-test was used in
1238 GraphPad Prism7. For pairwise comparisons, two-tailed unpaired *t*-tests were used in

1239 GraphPad Prism7. For multiple comparisons within one data set, one-way ANOVA with
1240 Dunnett's multiple comparison (comparing every mean with the control mean rather than
1241 comparing every mean with every other mean) was used in GraphPad Prism7. Mixed
1242 model statistics (this test does not assume normality or equal variance) was used with the
1243 experiment as random factor only in case confounding variation in baseline measurements
1244 between individual EC isolations (for each experiment, ECs were freshly isolated from
1245 individual human umbilicals) or mouse litters precluded the use of the above described
1246 statistical tests. For this, R and the *lme4* package were used; *P* values were obtained with
1247 the Kenward-Roger F-test for small mixed effect model datasets. In the most severe
1248 cases, the individual datapoints (each datapoint being the mean of the technical replicates
1249 within an experiment or an individual animal) in the bar graphs have been color-coded per
1250 experiment or per litter to show the baseline variation. Sample size for each experiment
1251 was not pre-determined. A *P* value <0.05 was considered significant.

1252 **DATA AVAILABILITY:** Fig.1, Fig. 4, Fig. 5, Extended Data Fig. 1, Extended Data Fig. 7 and
1253 Extended Data Fig. 8 have associated raw data (uncropped blots and/or gel pictures) in
1254 Supplemental Information Fig. 1. Fig. 1, Fig. 2, Extended Data Fig. 1 and Extended Data
1255 Fig. 4 have associated raw data (Excel files) for all bar graphs representing data from
1256 experiments involving mouse models. For the molecular modelling of palmitoyl-CoA
1257 docking into GS, models and trajectories are available on Figshare (doi:
1258 10.6084/m9.figshare.6575438). Any additional information required to interpret, replicate
1259 or build upon the Methods or findings reported in the manuscript is available from the
1260 corresponding author upon request.

1261

1262 REFERENCES UNIQUE TO THE METHODS SECTION

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1349 **LEGENDS TO EXTENDED DATA TABLES**

1350 **EXTENDED DATA TABLE 1: WEIGHT, HEMATOLOGICAL AND BLOOD PLASMA**
1351 **PARAMETERS FOR 6 WEEK-OLD GS^{VECKO} MICE AND CONTROL LITTERMATES**

1352 Values are mean±s.e.m. of n=14 (control) vs n=17 (GS^{VECKO}) animals. **P* = 0.0232 vs
1353 control, Student's *t* test. WBCs, white blood cells; RBCs, red blood cells, AST, aspartate
1354 amino transferase; ALT, alanine amino transferase; γ -GT, gamma-glutamyl transferase;
1355 CRP; C-reactive protein.

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1357 **EXTENDED DATA TABLE 2: ALIGNMENT OF THE AMINO ACID SEQUENCE**
1358 **ENCOMPASSING THE C209 RESIDUE ACROSS DIFFERENT SPECIES.**

1359 Multiple sequence alignment showing the conservation of amino acid C209 (in red) in GS
1360 across different species. Here the sequence alignment of 41 residues surrounding this
1361 cysteine is shown for up to 100 of the closest sequence identity matches with *Homo*
1362 *sapiens* GS obtained with BLAST from the UniProtKB/Swiss-Prot database. C209 is
1363 mostly conserved across species and when not conserved it is often substituted by
1364 residues (Ser or Thr) that can (in theory) be palmitoylated as well. In *Escherichia coli*
1365 (shown at the bottom), for example, a Thr is found at the structurally equivalent position
1366 (T210, highlighted in yellow). If for one and the same species multiple GS isoforms are
1367 known, only the one with the highest % identity is shown.

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1374 **LEGENDS TO EXTENDED DATA FIGURES**

1375 **EXTENDED DATA FIGURE 1: GS KNOCK-OUT IMPAIRS VESSEL SPROUTING**

1376 **a**, GS mRNA levels in human umbilical vein ECs (HUVEC; n=9 donors), lung ECs (n=5),
1377 colon ECs (n=4), liver ECs (n=3), human umbilical arterial ECs (HUAEC; n=2) and human
1378 blood outgrowth ECs (BOEC (n=2); (mean±s.e.m.; **P* <0.05 vs HUVEC, Student's *t* test)
1379 and in HEPG2 cells (mean±s.e.m.; n=3; **P* <0.05 vs HUVEC, Student's *t* test). **b-c**,
1380 Western blot of GS protein levels in HUVECs and HEPG2 cells in medium containing 0.6
1381 mM glutamine (+) or 0.025 mM glutamine (-) (b), and in isolated mouse liver ECs (mLiECs)

1382 and mouse astrocytes (c) (representative immunoblots of two independent experiments
1383 are shown). **d-e**, Genomic organization of the loxed *GS* allele before and after Cre-
1384 mediated excision (d) and correct recombination of the lox allele (L) in GS^{vECKO} and
1385 GS^{pECKO} mice upon tamoxifen (tam) treatment, as assessed by genomic DNA PCR (e; the
1386 PCR to amplify the loxed *GS* allele (lox) or to amplify the Cre-recombined allele (Δ) were
1387 run in separate reactions but loaded in the same lane; the gel picture shown is
1388 representative for all control, vECKO and pECKO mice used in this study). **f**, Quantification
1389 of branchpoints at the rear of the plexus in GS^{vECKO} mice (mean \pm s.e.m.; n=10 animals for
1390 GS^{vECKO} and 11 for wild-type (WT) controls from 3 litters; * P <0.05 vs WT littermates, mixed
1391 models R statistics). **g**, Pericyte coverage of retinal microvessels in WT and GS^{vECKO}
1392 littermates determined by NG2 staining and shown as NG2⁺ area as % of vessel area
1393 (mean \pm s.e.m.; n=4 animals for WT and 3 for GS^{vECKO} from 1 litter; ^{NS} P >0.05 vs WT,
1394 Student's *t* test). **h**, Reduced complexity of the retinal vascular front in P5 GS^{vECKO} vs WT
1395 animals determined by the number of branches on distal sprouts (mean \pm s.e.m.; n=13
1396 animals for WT and 21 for GS^{vECKO} from 5 litters; * P <0.05 vs WT, Student's *t* test). **i**,
1397 Quantification of EdU⁺ ECs at the rear of the plexus (mean \pm s.e.m.; n=12 animals for WT
1398 and 22 for GS^{vECKO} from 4 litters; ^{NS} P >0.05 vs WT littermates, Student's *t* test). **j-m**,
1399 Isolectin B4 staining of P5 retinal vascular plexi from WT (j) and GS^{pECKO} (k) mice
1400 (representative pictures with zoom-in insets, A=artery, V=vein) and quantification of branch
1401 points at the front (l) and the rear (m) of the plexus (mean \pm s.e.m.; n=10 animals for WT
1402 and 18 for GS^{pECKO} from 4 litters; * P ≤0.05 vs WT littermates, Student's *t* test). **n-u**,
1403 Isolectin B4 staining of the retinal microvasculature of 3 week (P21)-old (n,o) and 6 week
1404 (P42)-old (r,s) WT and GS^{vECKO} littermates (A=artery, V=vein). Lower left insets display
1405 higher magnification of IB4-stained superficial plexus, whereas lower right insets display
1406 higher magnification of the deep plexus. Also shown is the corresponding quantification of
1407 the vascular area (p,t) and the branch point density (q,u) in the superficial and the deep

1408 layer (mean±s.e.m.; n=8 animals for WT and 8 for GS^{VECKO} at P21, from two litters; n=10
 1409 animals for WT and 14 for GS^{VECKO} at P42, from four litters; ^{NS}P>0.05 vs WT, Student's *t*
 1410 test). **v-ag**, Representative micrographs of heart (v,z), liver (w,aa) and kidney (x,ab)
 1411 sections from WT and GS^{VECKO} littermates immunostained for the EC marker endoglin and
 1412 of lung (y,ac) sections immunostained for the EC marker CD34 and corresponding
 1413 quantifications of endoglin⁺ (ad, heart; ae, liver; af, kidney) or CD34⁺ (ag) vascular area
 1414 (mean±s.e.m.; n=5 animals (4 for heart) for WT and 7 (6 for heart) for GS^{VECKO}, from two
 1415 litters, ^{NS}P>0.05 vs WT, Student's *t* test). **ah-ai**, Images of flat-mounted retinas from
 1416 control (ah) and MSO-treated (ai) ROP mice (vaso-obiterated area in white). Images
 1417 shown are representative for 7 (ah) and 6 (ai) mice. Exact *P* values: (a) HUVEC vs lung
 1418 ECs: 0.0278; HUVEC vs colon ECs: 0.1086; HUVEC vs liver ECs: 0.3334; HUVEC vs
 1419 HEPG2: <0.0001; (f) <0.0001; (g) 0.3491; (h) <0.0001; (i) 0.8247; (l) 0.0012; (m) 0.050; (p)
 1420 superficial: 0.1218; deep: 0.1720; (q) superficial: 0.9995; deep: 0.4289; (t) superficial:
 1421 0.9792; deep: 0.6602; (u) superficial: 0.7979; deep: 0.1275; (ad) 0.9021; (ae) 0.2279; (af)
 1422 0.7647; (ag) 0.3614. Scale bars are 200 μm in j-k, n-o and r-s, 20 μm in v-ac and 1 mm in
 1423 ah-ai. HEPG2: hepatocellular carcinoma cells; mLiEC: mouse liver ECs; Tam: tamoxifen;
 1424 lox: loxed allele; Δ: recombined allele; NG2: chondroitin sulfate proteoglycan 4; Edu: 5-
 1425 ethynyl-2'-deoxyuridine. For gel source images, see Supplemental Information Fig. 1.

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1427 **EXTENDED DATA FIGURE 2: EFFECTS OF SILENCING AND PHARMACOLOGICAL INHIBITION**
 1428 **OF GS ON EC VIABILITY AND CENTRAL METABOLISM**

1429 **a**, GS mRNA levels in control ECs and ECs transduced with two different non-overlapping
 1430 shRNAs targeting GS (GS^{KD1} and GS^{KD2}; GS^{KD1} is used in the experiments in the main
 1431 manuscript and denoted as GS^{KD}) or transfected with scrambled siRNA (SCR) or siRNA
 1432 targeting GS (siGS). Data are expressed as % of the respective control, denoted by the
 1433 horizontal dotted line (mean±s.e.m.; n=28 independent experiments for GS^{KD1}, n=3

1434 independent experiments for GS^{KD2} and n=9 independent experiments for siGS; **P*<0.05
 1435 vs the respective control; one sample *t* test). **b-c**, Quantification of number of sprouts (b)
 1436 and total sprout length (c) for spheroid sprouting assays with GS^{KD} ECs and GS^{KD} ECs
 1437 expressing a shRNA-resistant *GS* mutant (rGS^{OE}) (mean±s.e.m.; n=3 independent
 1438 experiments; **P*<0.05 and ^{NS}*P*>0.05 vs control; ANOVA with Dunnett's multiple
 1439 comparison vs control). **d**, Viability of control and GS^{KD} ECs as measured by lactate
 1440 dehydrogenase (LDH) release assay (mean±s.e.m.; n=3 independent experiments;
 1441 ^{NS}*P*>0.05 vs control, one sample *t* test). **e**, Intracellular reactive oxygen species (ROS)
 1442 levels measured by CM-H₂DCFDA staining (mean±s.e.m.; n=3 independent experiments;
 1443 ^{NS}*P*>0.05 vs control, Student's *t* test). **f**, Energy charge measurement (([ATP] + 1/2[ADP]) /
 1444 ([ATP] + [ADP] + [AMP])) in GS^{KD} and control ECs (mean±s.e.m.; n=3 independent
 1445 experiments; ^{NS}*P*>0.05 vs control, Student's *t* test). **g**, Ratio of oxidized glutathione over
 1446 total glutathione levels (GSSG/(GSH+GSSG)) in GS^{KD} and control ECs (mean±s.e.m.; n=4
 1447 independent experiments; ^{NS}*P*>0.05 vs control, Student's *t* test). **h**, NADP/NADPH ratio in
 1448 GS^{KD} and control ECs (mean±s.e.m.; n=5 independent experiments; ^{NS}*P*>0.05 vs control,
 1449 one sample *t* test). **i-k**, Effect of GS^{KD} on major metabolic fluxes including glycolysis (i),
 1450 glucose oxidation (j) and glutamine oxidation (k) (mean±s.e.m.; n=3 independent
 1451 experiments for (i), n=5 for (j) and n=4 for (k); ^{NS}*P*>0.05 vs control, one sample *t* test). **l,m**,
 1452 Oxygen consumption rate (OCR) in control, MSO-treated and GS^{KD} ECs in basal state and
 1453 after injection of oligomycin, FCCP and antimycin A (l) (mean±s.e.m.; n=3 independent
 1454 experiments), and calculation of OCR_{BAS}, OCR_{ATP} and maximal respiration (m)
 1455 (mean±s.e.m.; n=3 independent experiments). Exact *P* values: (a) GS^{KD1}: <0.0001; GS^{KD2}:
 1456 <0.0001; siGS: <0.0001; (b) ctrl vs GS^{KD}: 0.0147; ctrl vs GS^{KD} + rGS^{OE}: 0.9824; (c) ctrl vs
 1457 GS^{KD}: 0.0083; ctrl vs GS^{KD} + rGS^{OE}: 0.6528; (d) 0.5717; (e) 0.8206 ; (f) 0.3715; (g) 0.4398;
 1458 (h) 0.9291; (i) 0.4691 (j) 0.6643 (k) 0.6786. AU: arbitrary units; CM-DCF: 5-(and-6)-
 1459 chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; FCCP: carbonyl

1460 cyanide-4-(trifluoromethoxy) phenylhydrazone; OCR_{BAS}: basal oxygen consumption rate;
1461 OCR_{ATP}: ATP-generating oxygen consumption rate; RFU: relative fluorescence units;
1462 MSO, methionine sulfoximine.

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1464 **EXTENDED DATA FIGURE 3: GS KNOCK-DOWN REDUCES EC MOTILITY**

1465 **a**, Wound closure in control and GS^{KD2} EC monolayer scratch assays with or without
1466 MitoC-pretreatment (mean±s.e.m.; n=7 and 5 independent experiments for with and
1467 without MitoC respectively; **P*<0.05 vs corresponding control; Student's *t* test). **b**,
1468 Quantification of lamellipodial area (% of total cellular area) in control and GS^{KD2} ECs
1469 (mean±s.e.m.; n=3 independent experiments; **P*<0.05 vs control; Student's *t* test). **c**,
1470 Wound closure in monolayer scratch assays with SCR- and siGS-transfected ECs
1471 (mean±s.e.m.; n=5 independent experiments; **P*<0.05 vs SCR; Student's *t* test). **d**,
1472 Quantification of lamellipodial area (% of total cellular area) in SCR- and siGS-transfected
1473 ECs (mean±s.e.m.; n=5 independent experiments; **P*<0.05 vs SCR; Student's *t* test). **e**,
1474 [³H]-Thymidine incorporation into DNA in SCR- and siGS-transfected ECs (mean±s.e.m.;
1475 n=3 independent experiments; ^{NS}*P*>0.05 vs SCR; Student's *t* test). Exact *P* values: (a) ctrl
1476 vs GS^{KD2}: 0.0290; ctrl vs GS^{KD2} + MitoC: 0.0223; (b) 0.0088; (c) 0.0407; (d) 0.0083; (e)
1477 0.4335.

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1479 **EXTENDED DATA FIGURE 4: EFFECTS OF GS SILENCING ON CYTOSKELETON AND**
1480 **BARRIER FUNCTION**

1481 **a-h**, Images of control (a,c,e,g) and GS^{KD} (b,d,f,h) ECs after staining for α-tubulin (a,b), F-
1482 actin (c,d) and nuclear staining (e,f); images shown are representative for 3 independent
1483 experiments. **i-k**, Representative images of phalloidin + Hoechst-stained liver ECs 6 hours
1484 after isolation from control (i) and MSO-treated (j) mice, and corresponding quantification

1485 of F-actin levels (k) (mean±s.e.m.; n=5 mice per group; * P <0.05 vs control, Student's t
1486 test). **l-n**, Representative images of phalloidin-stained (F-actin) confluent monolayer
1487 control (l) and GS^{KD} (m) ECs aligning a scratch wound, and quantification of F-actin levels
1488 (n) (mean±s.e.m.; n=5 independent experiments; * P <0.05 vs control, Student's t test). **o**,
1489 Quantification of the length of discontinuous and continuous VE-cadherin-stained junctions
1490 in control and GS^{KD} ECs (mean±s.e.m.; n=4 independent experiments; * P <0.05 vs control,
1491 Student's t test). **p**, Quantification of VE-cadherin gap size index in control and GS^{KD} EC
1492 monolayers (mean±s.e.m.; n=4 independent experiments; * P <0.05 vs control, Student's t
1493 test). **q-v**, Corresponding representative images of monolayer control and GS^{KD} ECs
1494 stained for VE-cadherin (q,r,u,v) and F-actin (s,t,u,v). Yellow arrows in (r) point to
1495 discontinuous VE-cadherin junctions and yellow asterisks indicate intracellular gaps. **w**,
1496 Quantification of transendothelial electrical resistance (TEER) in control and GS^{KD} EC
1497 monolayers (mean±s.e.m.; n=4 independent experiments; * P <0.05 vs control, Student's t
1498 test at each time point). **x-z**, Quantification (x) of Evans blue dye extracted from the ears of
1499 control and MSO-treated mice induced by topical application of mustard oil (n=4 mice for
1500 each condition, * P <0.05; Student's t test) and representative pictures of the Evans blue
1501 leakage into the ear tissue in control (y) and MSO-treated (z) mice. Exact P values: (k)
1502 0.0030; (n) 0.0036; (o) continuous ctrl vs GS^{KD}: 0.0005; discontinuous ctrl vs GS^{KD}:
1503 0.0005; (p) 0.0356; (w) 0.0181; (x) 0.0002. Scale bar is 20 μ m in a-h and in l-m and 10 μ m
1504 in i-j and in q-v. AU: arbitrary units.

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1506 **EXTENDED DATA FIGURE 5: ENZYMATIC ACTIVITY OF GS AND ITS ROLE IN EC MIGRATION**

1507 **a**, Scheme of ¹⁵NH₄⁺ labeling of glutamate and glutamine with unlabeled carbons (blue)
1508 and labeled nitrogens (red). **b**, ¹⁵N incorporation into glutamine (% isotope enrichment in
1509 m+1 and m+2, 30 min after adding ¹⁵NH₄⁺) in medium with dialyzed serum and different
1510 glutamine concentrations (mean±s.e.m.; n=3 independent experiments; ANOVA with

1511 Dunnett's multiple comparisons vs 4 mM; * $P < 0.05$). **c**, ^{15}N incorporation into glutamate (%
1512 isotope enrichment in $m+1$) and glutamine (% isotope enrichment in $m+1$ and $m+2$), 30
1513 min after adding increasing concentrations of $^{15}\text{NH}_4\text{Cl}$ (mean \pm s.e.m.; $n=3$ independent
1514 experiments). **d**, Scheme of glutamine labeling from $[\text{U-}^{13}\text{C}]$ -glutamate with unlabeled
1515 nitrogens (blue) and labeled carbons (red). **e**, Label contribution of $[\text{U-}^{13}\text{C}]$ -glutamate to
1516 intracellular glutamine at various glutamine concentrations (% isotope enrichment in
1517 glutamine and glutamate $m+5$, 30 min after adding the tracer) (mean \pm s.e.m.; $n=3$
1518 independent experiments; ANOVA with Dunnett's multiple comparisons vs 4 mM; * P
1519 < 0.05). **f**, Scheme for $[\text{U-}^{13}\text{C}]$ -glucose carbon contribution to glutamine with labeled
1520 carbons (red) and unlabeled carbons (blue). Incorporation is shown after one turn of the
1521 TCA cycle. **g**, Total contribution of $[\text{U-}^{13}\text{C}]$ -glucose carbons to α -ketoglutarate, glutamate
1522 and glutamine in ECs in medium with or without glutamine, 48 h after adding the tracer
1523 (mean \pm s.e.m.; $n=3$ independent experiments; * $P < 0.05$ vs total contribution in Gln at 0.6
1524 mM external Gln, ANOVA with Dunnett's multiple comparisons). **h**, ^{15}N incorporation into
1525 glutamine (% isotope enrichment in $m+1$ and $m+2$, 30 min after adding $^{15}\text{NH}_4^+$) in ECs and
1526 HEPG2 cells (mean \pm s.e.m.; $n=4$ independent experiments (ND=not detected)). **i**, ^{13}C -
1527 glutamine uptake kinetics in control, MSO-treated and GS^{KD} ECs and subsequent
1528 conversion to glutamate. See Methods for explanation of the different time points. Data are
1529 expressed as $m+5$ isotopomer, as percentage of the total intracellular glutamine (gln) or
1530 glutamate (glu) pool (mean \pm s.e.m.; $n=3$ independent experiments, except for 30 min
1531 where $n=1$ experiment; no statistical differences between control, MSO-treated and GS^{KD}
1532 were observed for glutamine nor for glutamate; ANOVA with Dunnett's multiple
1533 comparison vs control at each time point; no statistical analysis was performed at 30 min).
1534 **j**, ^{14}C -glutamine uptake in control and GS^{KD} ECs (mean \pm s.e.m.; $n=5$ independent
1535 experiments; $^{\text{NS}}P > 0.05$ vs control, one sample t test). **k**, Ratio of intracellular glutamine
1536 (Gln) over glutamate (Glu) levels in control and GS^{KD} ECs (mean \pm s.e.m.; $n=3$ independent

1537 experiments; ^{NS} $P > 0.05$ vs control, Student's *t* test). **I**, Velocity measurement of control and
 1538 GS^{KD} ECs at different glutamine (Gln) concentrations (mean±s.e.m.; n=4 independent
 1539 experiments; $*P < 0.05$ vs corresponding control, mixed models R statistics). **m-n**, Effect of
 1540 glutamine concentration on sprout number (m) and total sprout length (n) in control and
 1541 GS^{KD} spheroids (mean±s.e.m.; n=3 independent experiments; $*P < 0.05$ vs corresponding
 1542 control, mixed models R statistics). **o-p**, Number of sprouts per spheroid (o) and total
 1543 sprout length (p) in control and MSO-treated EC spheroids (mean±s.e.m.; n=3
 1544 independent experiments; $*P < 0.05$ vs control, paired Student's *t* test). **q-s**, Effect of MSO-
 1545 treatment on EC motility parameters: wound closure of MitoC-treated ECs (q)
 1546 (mean±s.e.m.; n=11 independent experiments; $*P < 0.05$ vs control, Student's *t* test),
 1547 lamellipodial area (r) (mean±s.e.m.; n=10 independent experiments; $*P < 0.05$ vs control,
 1548 paired Student's *t* test) and F-actin levels, 1 h after latrunculin wash-out (s) (mean±s.e.m.;
 1549 n=4 independent experiments; $*P < 0.05$ vs control, one-sample *t* test). **t**, [³H]-Thymidine
 1550 incorporation in control and MSO-treated ECs (mean±s.e.m.; n=3 independent
 1551 experiments; ^{NS} $P > 0.05$ vs control, one sample *t* test). Exact *P* values: (b) m+1 0.025 mM
 1552 vs m+1 4 mM: 0.0096; m+1 0.6 mM vs m+1 4 mM: 0.1206; m+2 0.025 mM vs m+2 4 mM:
 1553 0.0839; m+2 0.6 mM vs m+2 4 mM: 0.9921; (e) Glu m+5 0.6 mM vs Glu m+5 4 mM:
 1554 0.9372; Glu m+5 0.025 mM + MSO vs Glu m+5 4 mM: 0.0034; Glu m+5 0.025 mM vs Glu
 1555 m+5 4 mM: 0.0215; Gln m+5 0.6 mM vs Gln m+5 4 mM: 0.9297; Gln m+5 0.025 mM +
 1556 MSO vs Gln m+5 4 mM: 0.9961; Gln m+5 0.025 mM vs Gln m+5 4 mM: 0.0268; (g) α-keto
 1557 0.6 mM vs Gln 0.6 mM: 0.0001; Glu 0.6 mM vs Gln 0.6 mM: 0.0001; Gln 0 mM vs Gln 0.6
 1558 mM: 0.0285; (i) Gln 0.5 min: ctrl vs MSO: 0.4846; ctrl vs GS^{KD}: 0.5904; Gln 10 min: ctrl vs
 1559 MSO: 0.6709; ctrl vs GS^{KD}: 0.6910; Gln 20 min: ctrl vs MSO: 0.5896; ctrl vs GS^{KD}: 0.6784;
 1560 Glu 0.5 min: ctrl vs MSO: 0.9774; ctrl vs GS^{KD}: 0.8810; Glu 10 min: ctrl vs MSO: 0.0502;
 1561 ctrl vs GS^{KD}: 0.9598; Glu 20 min: ctrl vs MSO: 0.9782; ctrl vs GS^{KD}: 0.7783. (j) 0.6623; (k)
 1562 0.6704; (l) ctrl vs GS^{KD} 0.1 mM: 0.0054; ctrl vs GS^{KD} 0.6 mM: 0.0247 ctrl vs GS^{KD} 2 mM:

1563 0.0017; (m) ctrl vs GS^{KD} 0.6 mM and 10 mM: < 0.0001; (n) ctrl vs GS^{KD} 0.6 mM and 10
1564 mM: < 0.0001; (o) 0.0313; (p) 0.0075; (q) 0.0019; (r) 0.0116; (s) 0.0091; (t) 0.5110. α -keto:
1565 α -ketoglutarate; GDH: glutamate dehydrogenase; Glu: glutamate; GS: glutamine
1566 synthetase; Gln: glutamine; MSO, methionine sulfoximine; MitoC: mitomycin C.

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1568 **EXTENDED DATA FIGURE 6: RESCUING THE GS^{KD} PHENOTYPE *IN VITRO***

1569 **a**, Schematic representation of the DORA RHOA FRET biosensor, depicting from N- to C-
1570 terminal the circularly permuted RHOA effector protein kinase N (cpPKN), the dimeric
1571 circularly permuted Venus (dcpVen), the ribosomal protein-based linkers (L9), the dimeric
1572 Cerulean3 (dCer3) and RHOA. **b-m**, Representative images of control (b-d), MSO-treated
1573 (e-g), GS^{KD} (h-j) and RHOJ^{KD} (k-m) ECs after staining for F-actin (phalloidin)
1574 (b,d,e,g,h,j,k,m) and pMLC (c,d,f,g,i,j,l,m). **n**, Quantification of the pMLC-immunoreactivity
1575 (mean \pm s.e.m.; n=5 independent experiments; * P <0.05 vs control, one sample t test). **o-t**,
1576 Representative images of control (o,q,s) and GS^{KD} (p,r,t) EC spheroids treated with vehicle
1577 (o,p) or the ROCK inhibitors Y27632 (q,r) or fasudil hydrochloride (s,t). **u-v**, Quantification
1578 of the number of sprouts per spheroid (u) and sprout length (v) (mean \pm s.e.m.; n=3
1579 independent experiments; * P <0.05 and ^{NS} P >0.05 vs untreated control, ANOVA with
1580 Dunnett's multiple comparisons vs untreated control). **w**, Quantification of the lamellipodial
1581 area in vehicle- or fasudil hydrochloride-treated control and GS^{KD} ECs (mean \pm s.e.m.; n=6
1582 independent experiments; * P <0.05 and ^{NS} P >0.05 vs untreated control, ANOVA with
1583 Dunnett's multiple comparisons vs untreated control). **x**, Quantification of the lamellipodial
1584 area in vehicle-, ML7- or peptide 18-treated GS^{KD} and control ECs (mean \pm s.e.m.; n=4
1585 independent experiments of which 3 experiments included the ML7-treatment; * P <0.05 vs
1586 untreated control, ANOVA with Dunnett's multiple comparisons vs untreated control). **y**,
1587 Scratch wound closure in vehicle-, ML7- or peptide 18-treated GS^{KD} and control ECs
1588 (mean \pm s.e.m.; n=3 independent experiments; * P <0.05 vs untreated control, ANOVA with

1589 Dunnett's multiple comparisons vs untreated control). **z**, Fold-changes (vs untreated
 1590 control ECs) in F-actin levels from phalloidin-stained vehicle-, ML7- or peptide 18-treated
 1591 GS^{KD} ECs (mean±s.e.m.; n=4 independent experiments of which 3 included the peptide
 1592 18-treatment; **P*<0.05 vs untreated control, one sample *t* test). **aa**, Fold-changes (vs
 1593 untreated control ECs) in pMLC levels from pMLC-immunostained vehicle-, ML7- or
 1594 peptide 18-treated GS^{KD} ECs (mean±s.e.m.; n=4 independent experiments of which 3
 1595 included the peptide 18-treatment; **P*<0.05 vs untreated control, one sample *t* test. Exact
 1596 *P* values: (n) MSO: 0.0372; GS^{KD}: 0.0060; RHOJ^{KD}: 0.0051; (u) GS^{KD} vs ctrl: 0.0045; Fasu
 1597 vs ctrl: 0.9596; GS^{KD} + Fasu vs ctrl: 0.8857; (v) GS^{KD} vs ctrl: 0.0199; Fasu vs ctrl: 0.8309;
 1598 GS^{KD} + Fasu vs ctrl: 0.9327; (w) GS^{KD} vs ctrl: 0.0074; Fasu vs ctrl: 0.5906; GS^{KD} + Fasu
 1599 vs ctrl: 0.9900; (x) GS^{KD} vs ctrl: 0.0011; GS^{KD} + ML7 vs ctrl: 0.0079; GS^{KD} + pep.18 vs ctrl:
 1600 0.0017; (y) GS^{KD} vs ctrl: 0.0034; GS^{KD} + ML7 vs ctrl: 0.0022; GS^{KD} + pep.18 vs ctrl:
 1601 0.0040; (z) GS^{KD}: 0.0058; ML7: 0.0072; pep.18: 0.0888; (aa) GS^{KD}: 0.0369; ML7: 0.0021;
 1602 pep.18: 0.1672. Fasu., fasudil hydrochloride; pep. 18, peptide 18. Scale bar is 20 μm in (b-
 1603 m) and 100 μm in (o-t). For gel source images, see Supplemental Information Fig. 1.

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1605 **EXTENDED DATA FIGURE 7: RHOGTPASE LOCALIZATION AND INTERACTION WITH GS**

1606 **a**, Co-IP assays showing no detectable interaction between GS and RHOA or RHOC (red
 1607 asterisk indicates a non-specific band (also present in the IgG controls and unaffected by
 1608 silencing of RHOA or RHOC). Picture shown is representative for 3 independent
 1609 experiments. **b**, Co-IP of overexpressed GS and RHOJ-EGFP or ΔN-RHOJ-EGFP in ECs.
 1610 Quantifications are mean±s.e.m.; n=4 independent experiments; **P*<0.05, one-sample *t*
 1611 test. In some of the experiments, the expression of ΔN20-RHOJ-EGFP was lower than
 1612 expression of RHOJ-EGFP. To correct for this, densitometric quantification was performed
 1613 and signals in IP lanes were normalized to input signals. **c**, Immunoblotting for RHOA and
 1614 RHOC on cytosolic (c) and membrane (m) fractions of ECs with NaK as membrane marker

1615 and GAPDH as cytosolic marker. Picture shown is representative for 3 independent
1616 experiments. **d**, Bimolecular fluorescence complementation (BiFC) assay with GS coupled
1617 to the N-terminal half of EGFP, and RHOJ coupled to the C-terminal half of EGFP. Only
1618 when GS and RHOJ are in close proximity, the two EGFP half-sites complement each
1619 other and form a functional EGFP. **e**, Percentage of ECs displaying BiFC upon
1620 overexpression of GS-EGFP^{1/2} and RHOJ-EGFP^{2/2} or GS-EGFP^{1/2} and Δ N-RHOJ-
1621 EGFP^{2/2}. Data are mean \pm s.e.m.; n=3 independent experiments; **P*<0.05; Student's *t* test.
1622 **f**, Scheme for SPT-PALM imaging under TIRF illumination with the plasma membrane
1623 depicted at the top. The TIRF region is bright (whereas the part outside the TIRF region is
1624 grayed out) and contains the plasma membrane and its immediately adjacent space (not
1625 shown at exact relative dimensions). Weight and number of arrowheads represent velocity
1626 of single particles (the photoswitchable fluorescent protein (PSFP) or the PSFP coupled to
1627 the protein of interest (here GS)). The PSFP is activated upon entry into the TIRF region
1628 and is color-coded differently inside vs outside of the TIRF region. PSFP-GS displays
1629 reduced velocity in the TIRF region, presumably because of palmitoylation and membrane
1630 association of GS. **g**, Scheme for in-cell labeling of proteins with clickable alkyne-
1631 containing palmitoylation probes and subsequent biotin-azide clicking. X represents a
1632 palmitoylated protein, N₃ is the biotin-coupled azide. **h-i**, Rate of CoA release from
1633 palmitoyl-CoA as readout for recombinant human GS autopalmitoylation while varying
1634 either the doses of palmitoyl-CoA (h) or the amounts of recombinant GS (i) (mean \pm s.e.m.;
1635 n=4 independent experiments for h and n=5 for i; **P*<0.05, ANOVA with Dunnett's multiple
1636 comparisons vs 0 μ M palmitoyl-CoA or vs 0.5 μ g recombinant GS). **j**, Representative GS
1637 immunoblot (of 3 independent experiments) for binding of recombinant human GS to
1638 palmitoyl-CoA agarose. IF=input fraction; FT=flow through; W8=wash fraction 8;
1639 SDS=eluate. **k-m**, Representative images of RHOJ-EGFP localization in ECs under
1640 vehicle-treatment (k) or treatment with 2BP (pan-palmitoylation inhibitor) (l). Red

1641 arrowheads indicate EGFP signal at membrane ruffles, which was quantified as percent of
1642 total cellular area (m) (mean±s.e.m.; n=4 independent experiments; * P <0.05 vs vehicle-
1643 treated, paired Student's t test). **n-p**, Representative images of ECs overexpressing wt
1644 RHOJ-EGFP (n), RHOJ-EGFP point-mutated on cysteine residue 3 (C3A) (o) or RHOJ-
1645 EGFP point-mutated on cysteine residue 11 (C11A) (p). Red arrowheads indicate RHOJ at
1646 the plasma membrane. ECs that are not completely in the field of view have been masked
1647 out in blue. **q**, Quantification of the RHOJ-EGFP positive area at the plasma membrane as
1648 a percentage of total cell area. Data are mean±s.e.m.; n=5 independent experiments;
1649 * P <0.05; ANOVA with Dunnett's comparison vs wt RHOJ. **r**, RHOJ immunoblotting on
1650 membrane vs cytosolic fractions from ECs overexpressing wt RHOJ-EGFP (RHOJ WT),
1651 RHOJ-EGFP point-mutated on cysteine residue 3 (RHOJ C3A) or RHOJ-EGFP point-
1652 mutated on cysteine residue 11 (RHOJ C11A), with NaK as membrane marker and
1653 GAPDH and α -tubulin as cytosolic markers. **s**, Densitometric quantification of RHOJ/NaK
1654 as determined in (r). Data are mean±s.e.m.; n=6 independent experiments; * P <0.05; one
1655 sample t test. **t**, RHOJ activity in ECs under vehicle- or 2BP-treatment (blots are
1656 representative of 3 independent experiments; densitometric quantification in arbitrary units
1657 (AU) is mean±s.e.m.; * P <0.05, paired Student's t test vs vehicle-treated). **u**, RHOJ
1658 immunoblotting for control and GS^{KD} ECs overexpressing RHOJ (RHOJ^{OE}) subjected to
1659 acyl-RAC. The cleaved bound fraction (cBF) represents palmitoylated RHOJ. IF is the
1660 input fraction, whereas the cleaved unbound fraction (cUF) and the preserved bound
1661 fraction (pBF) are controls showing depletion of RHOJ from the thioester cleaving reagent
1662 and near absence of non-specific binding of RHOJ to the resin (see Methods).
1663 Densitometric quantification of cBF/IF is shown (mean±s.e.m; n=3 independent
1664 experiments; * P <0.05, one-sample t test vs control). **v**, GRAPHICAL ABSTRACT: *Left side*:
1665 Autopalmitoylation allows endothelial GS to interact directly (or indirectly) with the
1666 RhoGTPase RHOJ and to sustain RHOJ's palmitoylation, membrane localization and

1667 activity (reflected by GTP binding). RHOJ activity then sustains normal EC migration and
1668 lamellipodia formation, and keeps actin stress fiber formation at levels, promoting normal
1669 EC migration and vessel branching *in vivo*. Through mechanisms that are incompletely
1670 understood, active RHOJ inhibits signaling of the RHOA/B/C – ROCK – (p)MLC pathway
1671 (itself known to promote stress fiber formation). The relative contribution of a direct effect
1672 of RHOJ on migration vs the indirect effect through RHOA/B/C – ROCK – (p)MLC remains
1673 to be determined. Reduced opacity of RHOA/B/C, ROCK and (p)MLC indicates reduced
1674 signaling of this pathway. GTP: guanosine triphosphate. *Right side*: Loss of endothelial GS
1675 renders RHOJ less active (visually reflected by fewer palmitoylated, membrane-bound
1676 RHOJ proteins), and weakens the brake on the RHOA/B/C – ROCK – (p)MLC pathway.
1677 The resulting excessive stress fiber formation causes ECs to lose migratory capacity and
1678 reduces vessel branching *in vivo*. Dashed lines indicate reduced activity; red X indicates
1679 GS blockade; question mark indicates unknown mechanisms. Exact *P* values: (b) 0.0153;
1680 (e) 0.0334; (h) 2 vs 0 μ M: 0.6327; 5 vs 0 μ M: 0.2841; 10 vs 0 μ M: 0.1090; 20 vs 0 μ M:
1681 0.0339; 40 vs 0 μ M: 0.0034; (i) 1 vs 0.5 μ g: 0.5806; 2 vs 0.5 μ g: 0.0319; 4 vs 0.5 μ g
1682 :0.0037; 8 vs 0.5 μ g: 0.0001; 16 vs 0.5 μ g: 0.0001; (m) 0.0313; (q) RHOJ C3A vs RHOJ
1683 WT: 0.0001; RHOJ C11A vs RHOJ WT: 0.0001; (s) RHOJ C3A vs RHOJ WT: 0.0015;
1684 RHOJ C11A vs RHOJ WT: 0.0007; (t) 0.0051; (u) 0.0461. Scale bar is 200 μ m in k,l,n-p.
1685 For gel source images, see Supplemental Information Fig. 1.

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1687 **EXTENDED DATA FIGURE 8: POSSIBLE MOLECULAR MODEL OF GS AUTOPALMITOYLATION**

1688 **a.** Structure of human GS and of its bifunnel-shaped catalytic site. Schematic
1689 representation of the GS decamer in top and front view with individual subunits A and B
1690 labeled and colored gray and green, respectively. Close-up of the bifunnel catalytic site
1691 which is formed between subunits A and B. The GS decamer has 10 active sites, each
1692 located at the interface of two adjacent subunits. ATP enters from the top whereas

1693 glutamate enters from below; Manganese ions (Mn^{2+}) are shown as metallic spheres. **b.**
1694 Molecular dynamics (MD) simulation of palmitoyl-CoA in the catalytic cleft of GS predicts
1695 that, while the head of palmitoyl-CoA is tightly bound to the adenine binding site, the tail
1696 can point in opposing directions with respect to the protein's principal axis. The most
1697 representative structures of the two alternative poses observed during the long MD
1698 simulations for palmitoyl-CoA binding to GS (in blue, seen from two different perspectives)
1699 are shown in red (A, tail bending upwards) and green (B, tail bending downwards). **c.**
1700 Detailed view on the main conformation – conformation A – is shown in more details. The
1701 sulfur atom of palmitoyl-CoA (which is immediately adjacent to the carbon on which the
1702 nucleophilic attack occurs) (colored yellow) approaches the highly conserved C209 (also
1703 colored yellow), with an interatomic distance (S-S) that during the simulations reversibly
1704 fluctuates between 3 and 8 Å. The hydrophobic tail positions itself along grooves
1705 characterized by the presence of hydrophobic residues. Color coding: carbons are grey,
1706 nitrogens blue, phosphorous golden and oxygens red. Cysteines and serines within 5 Å
1707 from the palmitoyl tail are highlighted in yellow and orange, respectively. The hydrophobic
1708 residues around the tail are shown in green. **d.** Detailed view on conformation B where the
1709 tail is found in a buried hydrophobic cleft, with the sulfur at a distance of 5 Å or less from
1710 the conserved serines 65 and 75 and the tail occupying the site of the GS inhibitor MSO.
1711 Details are shown of the extensive steric clash between MSO and the secondary binding
1712 pose (B) observed in palmitoyl-CoA MD simulations. Palmitoyl-CoA is represented as
1713 sticks with standard atomic colours. MSO is shown in cyan and its position is taken from
1714 the 2QC8 entry in the protein databank. Cysteines and serines within 5 Å from the
1715 palmitoyl tail are highlighted in yellow and orange, respectively. The hydrophobic residues
1716 around the tail are shown in green. **e.** GS immunoblotting after streptavidin pull-down of
1717 biotin-azide clicked lysates from 16C-YA (palmitoylation probe) labeled HEK-293T cells
1718 overexpressing wild type GS or GS point-mutated for C209. The input shows the level of

1719 GS overexpression. Representative blot for 4 independent experiments is shown. **f-g.**
1720 Quantification of total sprout length (f) and number of sprouts per spheroid (g) for control
1721 and GS^{KD} ECs with or without overexpression of shRNA resistant C209A-point mutated
1722 GS (rGS^{C209A-OE}) (mean ± s.e.m.; n=4 independent experiments; **P* <0.05 vs control,
1723 ANOVA with Dunnett's multiple comparison vs control). **h.** Schematic representation of
1724 protein autopalmitylation. Upon binding of palmitoyl-CoA to the protein, free CoA (gray
1725 oval) is released and can be detected. **i.** Recombinant wild-type (WT) and point-mutated
1726 (R324C and R341C) GS were incubated with different doses of palmitoyl-CoA in a cell-free
1727 system at physiological pH. Release of CoA per minute was determined as a direct
1728 readout for protein autopalmitylation. Data are mean ± s.e.m. of 3 (R324C and R341C)
1729 and 4 (WT) independent experiments. ^{NS}*P*>0.05; **P*<0.05 according to two way ANOVA
1730 comparing the entire dose-response to the dose-response of WT GS. **j.** Different amounts
1731 of recombinant WT, R324C and R341C GS were incubated with a fixed amount of
1732 palmitoyl-CoA (40 μM) and CoA release per minute was determined as readout for
1733 autopalmitylation. Data are mean ± s.e.m. of 4 (R324C and R341C) and 5 (WT)
1734 independent experiments. ^{NS}*P*>0.05; **P*<0.05 according to two way ANOVA comparing the
1735 entire dose-response to the dose-response of WT GS. The data for WT GS from panels (i)
1736 and (j) are also included in Extended Data Fig. 7 as stand-alone data, but are included
1737 here too for comparison purposes. **k.** Boyden chamber migration for control, GS^{KD}, GS^{KD} +
1738 rGS^{OE} (r = shRNA-resistant; OE = overexpression), GS^{KD} + rGS^{R341C-OE} and GS^{KD} +
1739 rGS^{R324C-OE} ECs, all under mitomycin C-treatment (mean ± s.e.m.; n=3 independent
1740 experiments; ^{NS}*P*>0.05; **P*<0.05, ANOVA with Dunnett's multiple comparison vs control).
1741 Exact *P* values: (f) GS^{KD} vs ctrl: 0.0004; GS^{KD} + rGS^{C209A-OE} vs ctrl: 0.0004; (g) GS^{KD} vs
1742 ctrl: 0.0001; GS^{KD} + rGS^{C209A-OE} vs ctrl: 0.0001; (i) R324C vs WT: 0.8228; R341C vs WT:
1743 0.7530; (j) R324C vs WT: 0.1331; R341C vs WT: 0.0003; (k) GS^{KD} vs ctrl: 0.0054; GS^{KD} +

1744 rGS^{OE} vs ctrl: 0.8152; $GS^{KD} + rGS^{R341C-OE}$ vs ctrl: 0.3645; $GS^{KD} + rGS^{R324C-OE}$ vs ctrl:
1745 0.2118. For gel source images, see Supplemental Information Fig. 1.

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