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4 **Latrophilin-2 mediates fluid shear stress mechanotransduction at endothelial**
5 **junctions**
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9 Keiichiro Tanaka^{1,7,*}, Minghao Chen^{1,7}, Andrew Prendergast¹, Zhenwu Zhuang¹, Ali Nasiri²,
10 Divyesh Joshi¹, Jared Hintzen¹, Minhwan Chung¹, Abhishek Kumar¹, Arya Mani¹, Anthony
11 Koleske³, Jason Crawford⁴, Stefania Nicoli¹ and Martin A. Schwartz^{1,5,6,*}

12
13 ¹ Yale Cardiovascular Research Center, Section of Cardiovascular Medicine, Department of
14 Internal Medicine, School of Medicine, Yale University, New Haven, CT 06511, USA

15 ² Department of Internal Medicine

16 ³ Department of Molecular Biochemistry and Biophysics

17 ⁴ Department of Chemistry

18 ⁵ Department of Cell Biology

19 ⁶ Department of Biomedical Engineering

20 ⁷ These authors contributed equally.

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22 Running title: Latrophilins in shear stress sensing
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24 *Authors for correspondence:
25 keiichiro.tanaka@yale.edu; martin.schwartz@yale.edu;
26

27 **Abstract**

28 Endothelial cell responses to fluid shear stress from blood flow are crucial for vascular
29 development, function and disease. A complex of PECAM-1, VE-cadherin, VEGF receptors
30 (VEGFRs) and PlexinD1 located at cell-cell junctions mediates many of these events. But
31 available evidence suggests that another mechanosensor upstream of PECAM-1 initiates
32 signaling. Hypothesizing that GPCR and G α proteins may serve this role, we performed siRNA
33 screening of G α subunits and found that G α i2 and G α q/11 are required for activation of the
34 junctional complex. We then developed a new activation assay, which showed that these G
35 proteins are activated by flow. We next mapped the G α residues required for activation and
36 developed an affinity purification method that used this information to identify latrophilin-2
37 (Lphn-2/ADGRL2) as the upstream GPCR. Latrophilin-2 is required for all PECAM-1
38 downstream events tested. In both mice and zebrafish, latrophilin-2 is required for flow-
39 dependent angiogenesis and artery remodeling. Furthermore, endothelial specific knockout
40 demonstrates that latrophilin plays a role in flow-dependent artery remodeling. Human genetic
41 data reveal a correlation between the latrophilin-2-encoding *Adgrl2* gene and cardiovascular
42 disease. Together, these results define a pathway that connects latrophilin-dependent G protein
43 activation to subsequent endothelial signaling, vascular physiology and disease.

44

45 **Keywords**

46 Latrophilin, G protein-coupled receptor, fluid shear stress, vascular development, PECAM-1
47

48 **Introduction**

49 Fluid shear stress (FSS) from blood flow is critical for early vascular development in
50 mice(Lucitti *et al*, 2007) and zebrafish(Sugden *et al*, 2017), for homeostatic vessel remodeling in
51 adults(Humphrey & Schwartz, 2021; Vogel *et al*, 2020), and in the initiation and progression of
52 atherosclerosis(Baeyens *et al*, 2016; Zhou *et al*, 2014). A complex of proteins at cell-cell
53 junctions consisting of PECAM-1, VE-cadherin, VEGFR2 and 3, and Plexin D1 plays an
54 important role in shear stress responses including vasodilation, flow-dependent vessel
55 remodeling, and atherosclerosis(Conway & Schwartz, 2012; Givens & Tzima, 2016).
56 Stimulation of this junctional complex by flow triggers activation of Src-family kinases (SFKs)
57 within seconds, resulting in ligand-independent activation of VEGF receptors and downstream
58 events including endothelial cell (EC) alignment in the direction of flow(Collins *et al*, 2012).
59 However, the mechanisms by which proteins located at cell-cell junctions can sense forces from
60 shear stress exerted on the apical surface is unclear. Previous work suggests that activation of the
61 junctional complex is not primary but rather requires an upstream event, mediated presumably by
62 another mechanosensor (Conway *et al*, 2013).

63 GPCR/G α protein signaling is an obvious candidate for initiating signaling on this time scale
64 (seconds). GPCRs are a large family of cell surface receptors that play a crucial role in
65 transducting signals across cell membranes. GPCRs undergo conformational changes in
66 response to ligand binding or other stimuli, after which the activated intracellular domain binds
67 to a heterotrimeric G α protein complex and catalyzes the exchange of guanosine diphosphate
68 (GDP) for guanosine triphosphate (GTP). Connections between GPCRs and shear stress have
69 been reported(Chachisvilis *et al*, 2006; Dela Paz & Frangos, 2019; Erdoganmus *et al*, 2019; Jung *et*
70 *al*, 2012; Wang *et al*, 2015; Xu *et al*, 2018). However, the literature on G α proteins and GPCRs
71 in shear stress signaling is highly inconsistent(Chachisvilis *et al.*, 2006; Dela Paz & Frangos,
72 2019; Jung *et al.*, 2012; Wang *et al.*, 2015). For instance, it has been demonstrated that flow
73 applied to endothelial cells activates G α proteins independent of an intervening GPCR(Dela Paz
74 *et al*, 2017; Gudi *et al*, 1998). It has also been reported that shear stress responses related to

75 PECAM-1 and cell-cell junction require GPCRs but with different GPCRs implicated in different
76 studies. These studies include reports of ligand-independent activation of a bradykinin
77 receptor(Chachisvilis *et al.*, 2006; Yeh *et al*, 2008) and the sphingosine 1-phosphate receptor
78 S1PR1(Jung *et al.*, 2012). The purinergic receptor P2Y2 was also implicated, in this case via
79 ATP release downstream of activation of the mechanosensitive ion channel Piezo1(Albarran-
80 Juarez *et al*, 2018; Wang *et al.*, 2015), though this pathway has been disputed(Dela Paz &
81 Frangos, 2019). The orphan receptor GPR68 was identified as a shear stress sensor specific to
82 small arteries though the mechanism of activation, including ligand dependence or independence,
83 is unknown(Xu *et al.*, 2018). These studies also vary in the G α proteins implicated, which
84 included different G α i isoforms (Gudi *et al*, 1996; Jung *et al.*, 2012) and the G α q/11
85 family(Albaran-Juarez *et al.*, 2018; Chachisvilis *et al.*, 2006; Wang *et al.*, 2015).

86 To resolve this issue, we took an unbiased approach starting from the G α proteins using the
87 dramatic morphological changes in endothelial alignment to flow as the initial readout. Our
88 results showed that G α i2 and G α q/11 function in parallel, identified latrophilin-2 (LPHN2) as the
89 key upstream GPCR and demonstrated a specific requirement for LPHN2 in flow-dependent
90 remodeling in zebrafish and mice.
91

92 **Results**

93 Identification of G α protein subunits required for flow sensing

94 To investigate the roles of GPCR signaling in endothelial flow responses, we first
95 transfected human umbilical vein endothelial cells (HUVECs) with short interfering RNAs
96 (siRNAs) to suppress Gs, Gi, Gq/11 and G12/13 classes of G α proteins and assayed EC
97 alignment in flow as our initial readout. Single knockdowns gave either weak (Gi) or no (Gs,
98 G12/13, Gq/11) inhibition of EC alignment in flow (Fig.1A and Appendix fig. S1A-C). However,
99 simultaneous knockdown of Gi and Gq/11 completely inhibited alignment (Fig. 1A), whereas
100 other combinations were ineffective (Appendix fig. S1D, S1E). Activation of SFKs is the earliest
101 flow-responsive event mediated by PECAM-1, followed by VEGFRs and Akt(Tzima *et al*, 2005).
102 These events were similarly blocked by knockdown of Gi plus Gq/11 but not by single
103 knockdowns (Fig. 1B, 1C and Appendix fig. S1F). Therefore, Gi and Gq/11 act in parallel to
104 initiate PECAM-1-dependent flow signaling.

105 Rescue controls with siRNA-resistant G α variants showed that siRNA-resistant Gq and Gi2
106 restored cell alignment, whereas Gi1 and Gi3 did not (Fig. 1D and Appendix fig. S2A, S2B).
107 This unexpected result prompted us to search for residues that are similar in Gq and Gi2 but
108 different in Gi1 and Gi3. Two residues (using human Gi2 numbering) fit this pattern: D167 in
109 Gi2 corresponds to N in Gi1/3; and K307 corresponds to Q306 in Gi1/3 (Fig. 1E). Gi1(Q306K),
110 but not Gi1(N166D), rescued EC alignment in flow (Fig. 1F and Appendix fig. S2C).
111 Consistently, Gi2 mutant lacking K307 (K307Q) could not rescue the defects on flow-induced
112 alignment under the knockdown of Gi plus Gq/11 (Appendix fig. S1G and Appendix fig. S2D).
113 Therefore, K307 in Gi2 distinguishes the G α subunits participate in flow signaling.

114

115 Flow-induced activation of specific G α proteins

116 K307 lies in the region that specifies coupling with GPCRs(Bae *et al*, 1999; Flock *et al*,
117 2017), suggesting that K307 may determine G α activation. However, testing this hypothesis
118 requires measurement of G α activation in live cells. Unfortunately, indirect G α activation assays

119 using second messengers such as cAMP or calcium are unreliable in the context of fluid shear
120 stress where multiple activating/suppressing pathways are stimulated. We therefore developed
121 pulldown assays for G α based on the specific binding of GTP-loaded G α subunits to effector
122 proteins, namely GINIP for Gi and GRK2 N-terminal domain for Gq (Appendix fig.
123 S3a)(Gaillard *et al*, 2014; Tesmer *et al*, 2005). To facilitate these assays and distinguish specific
124 G α isoforms (especially Gi isoforms), we prepared versions containing an internal GluGlu (EE)
125 epitope tag that does not impair function (Appendix fig. S3B and refs(Medina *et al*, 1996;
126 Wilson & Bourne, 1995)). For Gq, to enhance pulldown efficiency, we co-expressed Ric8A,
127 which stabilizes active Gq(Papasergi *et al*, 2015). Active, but not inactive, Gi and Gq bound to
128 these effector proteins, while when the artificial GPCR DREADD was expressed, its ligand
129 clozapine-N-oxide (CNO) also activated G α proteins (Appendix fig. S3C-S3F). Importantly, this
130 G α pulldown assay showed not only rapid activation of Gi2 and Gq by FSS (Fig. 1G-J), but also
131 differential responses of Gi1 and gain-of-function Gi1(Q306K) to FSS (Fig. 1K, 1L). Gi
132 activation was unaffected by PECAM-1 knockdown, indicating that this step was upstream or
133 independent of PECAM-1 (Fig. 1M, 1N). Flow-mediated activation of specific G α subunits thus
134 corresponds to their requirement in EC flow responses.

135

136 Identification of the upstream GPCR

137 We next sought to identify the GPCR(s) that mediates flow activation of Gi2 and Gq11.
138 Although affinity purification is the obvious choice, rapid release of G α proteins from GPCRs
139 after GTP loading limits this approach. Starting with wild-type and its Q306K mutant, we
140 inserted 1) an internal GFP for affinity purification and 2) four alanines (hereafter ins4A) into
141 helix α 5, which blocks GTP loading, thereby preventing dissociation from GPCRs even in the
142 presence of GTP(Kaya *et al*, 2016) (Fig. 2A). Both constructs localized mainly to the plasma
143 membrane, as did wild-type G α (Appendix fig. S4A). We further employed detergent-free
144 nanodisc-forming styrene-maleimide anhydride (SMA) copolymer to stabilize GPCR
145 conformation within its native lipid environment(Lee *et al*, 2016). This approach was validated

146 by coimmunoprecipitation using GFP-Trap® nanobody beads of G α variants with DREADD
147 GPCR, which showed increased association after receptor activation (Appendix fig. S4B, S4C).
148 Proteomic analysis for GPCRs that associated with Gi1(Q306K) but not wild-type Gi1 in
149 response to FSS (protocol in Appendix fig. S4D), identified S1PR1 and ADGRL3 (Appendix fig.
150 S4E). However, depletion of S1PR1 did not block alignment in FSS (Appendix fig. S4F, S4G).
151 Furthermore, S1PR1 reportedly activates Gi1 and Gi3 as well as Gi2(Lee *et al*, 1996), which is
152 inconsistent with the G α specificity defined above. We therefore focused on ADGRL family
153 proteins, also called latrophilins (LPHNs).

154 Latrophilins (1,2 and 3 in mammals) are adhesion-type GPCRs that bind to counterreceptors
155 on neighboring cells and regulate neuronal synapses(Sando *et al*, 2019). Although it has been
156 reported that different types of endothelial cells have different latrophilin isoforms, LPHN2 is
157 most widely expressed and is the overwhelming major isoform in HUVECs (Appendix figs
158 S5A)(Maleszewska *et al*, 2016). Consistent with the proteomic results, Gi1(Q306K)(ins4A)
159 associated with endogenous LPHN2, which increased upon FSS (Fig. 2B, C). This result
160 strongly suggests that FSS activates LPHN2 to recruit G α proteins. Furthermore, Gi1(Q306K),
161 Gi2, and Gq co-immunoprecipitated with LPHN2, whereas Gi1 did not, indicating that LPHN2
162 specifically bound to the flow-responsive G α proteins defined above (Fig. 2D). Importantly,
163 LPHN2 knockdown blocked flow-mediated activation of Gi2 (Fig. 2E, 2F). Together, these data
164 provide strong evidence that latrophilin-2 mediates G α activation essential for endothelial
165 alignment in response to flow.

166

167 Latrophilins in EC flow signaling *in vitro*.

168 Knockdown of LPHN2, but not LPHN1 or LPHN3, blocked EC alignment in flow (Fig. 3A,
169 3B; knockdown confirmed in Appendix fig. S5B, S5C). LPHN2 knockdown in human aortic
170 ECs similarly blocked flow-induced alignment (Appendix fig. S5D, S5E). In line with these
171 results, LPHN2 knockdown also blocked Golgi apparatus polarization in flow (Appendix fig.
172 S5F, S5G). LPHN2 localized to cell-cell contacts (Fig.3C and Appendix fig. S6). The flow

173 responsive G1 mutant, Gi1(Q306K), also associated with PECAM-1 following FSS (Fig. 3D).
174 The association between LPHN2-Gi1(Q306K) complex with junctional mechanosensory
175 complex proteins PECAM-1 and VE-Cadherin was detected after 24 hours of flow (Fig. 3D-3G),
176 indicating a persistent interaction. We also assessed the immediate flow-dependent signals.
177 LPHN2 depletion strongly suppressed flow-mediated acute activation of SFKs and Akt (Fig 3H-
178 3J). Thus, LPHN2 colocalizes, physically associates with and is required for signaling through
179 the junctional complex. However, LPHN2 knockdown did not inhibit activation of VEGFR2 by
180 VEGF-A, indicating that LPHN2 functions specifically in response to laminar flow (Appendix
181 fig. S7A, S7B).

182 Among the 3 latrophilin isoforms in human genome, all rescued flow-mediated endothelial
183 alignment after LPHN2 knockdown (Fig. 3K, expression confirmed in Appendix fig. S8A),
184 indicating functional equivalence; the observed differences are therefore due to differential
185 expression. EC alignment was rescued by wild type (WT) LPHN2 but not H1071A, which is
186 defective in coupling to G α proteins(Nazarko *et al*, 2018) (Fig. 3L, expression confirmed in
187 Appendix fig. S8B). Adhesion-type GPCRs can be activated by internal cleavage by the
188 extracellular GAIN domain which exposures its *Stachel* peptide as a tethered agonist(Vizurraga
189 *et al*, 2020). However, the autoproteolysis-defective mutant fully rescued EC alignment (see
190 Fig.3L). Helix-8 is known to endow some GPCRs with mechanosensitivity to membrane
191 stretch(Erdogmus *et al.*, 2019). However, a LPHN2 helix-8 mutant could still rescue EC
192 alignment in flow (Fig. 3L). Taken together, these findings indicate that latrophilin regulates G α
193 activation required for endothelial flow responses, but activation requires neither the *Stachel*
194 peptide nor helix-8.

195

196 Latrophilin-2 regulates flow-induced endothelial cell morphological changes *in vivo*

197 Long-term laminar flow stabilizes EC junctions, promoting a linear, continuous morphology
198 associated with barrier function(Kroon *et al*, 2017) (see Fig.3A). To test whether the role of
199 LPHN2 in this process, we analyzed junctional morphology *in vitro* with and without 16h of

200 flow. In control cells, shear stress shifted junctions from an irregular morphology to linear
201 junctions; in Lphn2 knockdown ECs, junctions were indistinguishable in the absence of flow but
202 failed to linearize in flow (Fig. 4A, 4B), demonstrating that the effect is flow-specific. To further
203 examine contributions of blood flow/latrophilin-2 to ECs *in vivo*, zebrafish embryos are useful as
204 a developmental system that is amenable to manipulation of shear stress using morpholinos
205 against cardiac troponin T type 2a (*tnnt2a*; ‘*silent heart*’)(Sehnert *et al*, 2002), without global
206 adverse effects. We employed zebrafish embryos expressing Tg(*kdr1:ras-mCherry*) to mark
207 endothelial cells(Sehnert *et al.*, 2002) and designed sgRNAs for CRISPR-Cas9 targeting all
208 latrophilin isoforms (*adgrl1*, *adgrl2a* plus *adgrl2b.1*, and *adgrl3*). Our sgRNAs induced >50%
209 cleavage in the T7 endonuclease assays and reduced mRNA levels through nonsense-mediated
210 decay (Appendix fig. S9). Since Lphn2 is the major endothelial isoform, we performed CRISPR-
211 mediated F(0)/mosaic knockout of *adgrl2a+adgrl2b.1* (hereafter Lphn2 mutation), which
212 inhibited elongation and enlargement of ECs in the dorsal aorta (Fig. 4C). Injecting single cell
213 embryos with *silent heart* morpholinos at a dose that completely blocked cardiac contractility
214 similarly blocked the elongation of ECs. Furthermore, combining *tnnt2a* and Lphn2 mutation had
215 no further effect, supporting a specific role for latrophilins in flow sensing (Fig. 4D, 4E).

216 To address LPHN function in mammals, we crossed Lphn2^{flox/flox} mice with CDH5-Cre^{ERT2}
217 mice (Lphn2 ECKO), which were injected with tamoxifen at 8 weeks of age and remained viable
218 10 weeks later. The descending thoracic aorta (a region of high laminar flow) of Lphn2 ECKO
219 mice had poorly aligned ECs with irregular junctions (Fig. 4F-H). Additionally, Lphn2 ECKO
220 increased vascular permeability in the whole aorta, as assessed by Evans blue extravasation (Fig.
221 4I, 4J). *In vitro*, flow for 24h increased HUVEC barrier function, which was blocked by LPHN2
222 knockdown, whereas barrier function in the absence of flow was unaffected (Fig. 4K, 4L).
223 Latrophilins are therefore flow-responsive GPCRs that regulate multiple steps in PECAM1-
224 dependent EC mechanotransduction.

225

226 Plasma membrane fluidization initiates latrophilin signaling

We next considered how FSS activates LPHN2. As mutation of the autoproteolytic GAIN domain, which is speculated to be mechanosensitive, had no effect (Figure 3i), we considered alternatives. FSS induces a rapid increase in plasma membrane fluidity, in some cases associated with decreased plasma membrane cholesterol(Butler *et al*, 2001; Haidekker *et al*, 2000; Yamamoto & Ando, 2013; Yamamoto *et al*, 2020). As GPCRs can respond to such changes(Vizurraga *et al.*, 2020), HUVECs were treated with methyl- β -cyclodextrin (M β CD) to extract plasma membrane cholesterol. Treatment with 5 mM M β CD for 1 min reduced plasma membrane cholesterol to the same extent as flow, assayed by binding of the cholesterol-specific probe D4H-Clover3(Liu *et al*, 2017) (Figure 5A). No discernable effect on cytoskeletal organization was observed under these conditions (Figure 5B). M β CD treatment also induced Ga binding to LPHN2 (Figure 5C) and activated both SFKs and VEGFR2, which were blocked by LPHN2 knockdown (Figure 5D-F). Together with published data demonstrating that cholesterol supplementation blocks flow signaling to these and related pathways(Fancher *et al*, 2018; Tirziu *et al*, 2005; Yamamoto & Ando, 2013, 2015; Yamamoto *et al.*, 2020), these results suggest that membrane fluidization can trigger LPHN2 GPCR activation and downstream signaling (Figure 5G).

243

244 Latrophilins in vascular remodeling *in vivo*

To address roles of LPHN2 in flow-dependent vascular remodeling, we examined zebrafish embryos, where artery lumen diameters are determined by EC shear stress(Sugden *et al.*, 2017). We observed that intersegmental vessels (ISVs) at 48 hours post fertilization had reduced diameters after both latrophilin knockout and blockade of blood flow, with no further effect when combined (Fig. 6A, 6B). At 72 hpf, when intersegmental arteries and veins are distinguishable, the decrease in diameter was observed only in arteries (Fig. 6C). LPHNs are thus required for effects of flow on artery diameter.

These results prompted us to examine flow-dependent vascular remodeling in mice. Femoral artery ligation triggers flow-driven arteriogenesis in the thigh and hypoxia-driven

254 angiogenesis in the calf^(Cornella *et al*, 2017). Following surgery, LPHN2 ECKO mice showed
255 lower blood flow in the foot even at day 1 and markedly slower recovery than WT littermates
256 (Fig. 6D, 6E). To understand these effects, we performed micro-CT of vascular casts after
257 injection of electron-dense bismuth microparticles that label arteries/arterioles but not capillaries
258 due to particle size. Surgery induced an increase in small arteries in WT mice but essentially
259 none in LPHN2 ECKO mice (Fig. 6F, 6G). LPHN2 ECKO mice also had much lower density of
260 small arteries even in the control leg, consistent with lower flow on day 1. Staining for PECAM
261 to examine the total vasculature in leg muscle sections revealed decreased capillary density as
262 well, even at baseline (Fig. 6H, 6I). LPHN2 ECKO mice thus show low vascular density at
263 baseline and greatly reduced flow-dependent remodeling. As expected from the low vascular
264 density, a treadmill fatigue test (Fig. 6J) LPHN2 ECKO mice showed markedly lower exercise
265 capacity (Fig. 6K, 6L) and impaired oxygen consumption (Fig. 6M). LPHN2 ECKO thus results
266 in vascular defects *in vivo*, at least in part due to defective EC flow sensing. To address whether
267 these defects were related to shear stress sensing, we examined an *in vitro* system in which FSS
268 enhances VEGF-induced sprouting into collagen gels(Galie *et al*, 2014). Enhanced sprouting was
269 completely blocked by LPHN2 depletion with only a weak effect on basal migration (Fig. 6N-
270 6P).

271

272 Genetic link to human disease.

273 The Cardiovascular Disease Knowledge Portal reported four SNPs near the 5' end of the
274 *ADGRL2* gene (Appendix fig. S10A) that exhibit genome wide association with hypertension
275 (Appendix fig. S10B). All 4 are in close proximity to each other and are in linkage
276 disequilibrium (D' : 0.9953, R^2 : 0.97). Two of the SNPs meet the significant threshold ($p < 5 \times 10^{-8}$)
277 and rs1195871 is relatively conserved among vertebrates. Of particular interest, the intronic
278 SNP rs186892211 in the *ADGRL2* locus (Appendix fig. S10A) is associated with large-artery
279 atherosclerosis and subsequent ischemic stroke (TOAST classification) with a very high odds
280 ratio (odds ratio 720; $p < 3.134e-10$, Appendix fig. S10B).

281 **Discussion**

282 These data elucidate a pathway in which fluid shear stress acts through the latrophilin
283 adhesion GPCRs to activate subsequent cellular signaling events and vascular remodeling *in vivo*,
284 thus connecting time scales from seconds to hours to weeks/months. Previous studies showed
285 that laminar flow induces an increase in plasma membrane fluidity associated with depletion of
286 membrane cholesterol (Yamamoto & Ando, 2013, 2015; Yamamoto *et al.*, 2020). Our data
287 demonstrate that artificial cholesterol depletion activates flow-induced signals in a Lphn2-
288 dependent manner, suggesting that latrophilins connect physical changes in the plasma
289 membrane to cellular signaling. Latrophilins mediate activation of the PECAM-1/VE-
290 cadherin/VEGFR pathway through both Gi2 and Gq/11, suggesting parallel or redundant
291 downstream pathways. Consistent with these functional effects, FSS induces physical
292 interactions (direct or indirect) between LPHN2-G α and PECAM-1. However, LPHNs are not
293 required for activation of VEGFRs by its ligand VEGF.

294 Previous studies identified either Gi or Gq/11 as mediators of flow signaling(Chachisvilis *et*
295 *al.*, 2006; Jung *et al.*, 2012; Wang *et al.*, 2015). It seems plausible that, depending on EC
296 subtype or experimental conditions, either Gi or Gq/11 predominated, which concealed their
297 functional redundancy. Previous studies also identified other GPCRs as mediators of EC shear
298 stress responses, via direct or indirect mechanisms, as well as making claims for GPCR-
299 independent activation of G proteins(Dela Paz *et al.*, 2017). GPCRs implicated in shear stress
300 signaling include bradykinin receptor B2(Chachisvilis *et al.*, 2006), ATP receptor
301 P2Y2(Albarran-Juarez *et al.*, 2018; Wang *et al.*, 2015), sphingosine phosphate receptor
302 S1PR1(Jung *et al.*, 2012), the apelin receptor APJ(Strohbach *et al.*, 2018) and GPR68(Xu *et al.*,
303 2018). An indirect association of Gq/11 with PECAM1 was also reported, which dissociated
304 upon application of shear stress(dela Paz *et al.*, 2014). Differences in EC location or subtype
305 likely account for some of these effects. For example, GPR68 is expressed specifically in small
306 resistance arteries where it controls vasodilation but had no reported effect on development or
307 patterning(Xu *et al.*, 2018). P2Y2 was also reported to affect vasodilation and blood pressure

308 without reported effects on development or patterning. It seems likely that differences in local
309 expression may thus explain some of the apparent multiplicity, though additional variables such
310 as modulation by other inputs and coupling to different downstream effectors may also
311 contribute. Understanding this GPCR-G α protein signaling network is therefore an important
312 though complex direction for future work. The novel pulldown assay and GPCR-G α protein
313 affinity purification protocol developed here may facilitate such efforts.

314 It is also noteworthy that latrophilins are required only for activation of signaling events
315 including VEGFR2 by flow, but not for activation of VEGFR2 by its ligand, VEGF. PECAM-1
316 first appears in vertebrates but has multiple other functions in leukocyte transmigration and
317 activation, platelet reactivity and angiogenesis that are independent of flow(Privratsky *et al*,
318 2010). Together, these findings argue that latrophilins, in essence, confer flow sensitivity to pre-
319 existing pathways controlled by ligand availability.

320 Lphn2 mutation in zebrafish and endothelial knockout in mice caused multiple vascular
321 defects that implicate FSS mechanotransduction, including reduced EC alignment in the
322 direction of flow, altered junctional morphology and permeability, smaller artery diameters and
323 defects in arterialization in the hindlimb ischemia model. Lower capillary and small artery
324 density in some vascular beds may also be attributable to defective FSS sensing, as FSS regulates
325 angiogenic sprouting (Galie *et al*, 2014) and stabilizes nascent vessels once blood begins to flow
326 (Campinho *et al*, 2020). We note, however, that flow-independent functional roles for Lphn2 in
327 ECs have been observed (Camillo *et al*, 2021) and likely contribute as well.

328 These results link together many observations to provide a comprehensive model for EC
329 responses to FSS (Appendix fig. S11). These findings are of further interest in light of human
330 genetic data that strongly link the *Adgrl2* gene to vascular disease (Appendix fig. S10). However,
331 the identified polymorphisms reside in regulatory or intronic regions, thus, causal connections to
332 vascular disease are not obvious. Intronic polymorphisms can regulate gene expression but could
333 also be in linkage disequilibrium with causal SNPs that are not yet identified. More detailed
334 population genetic analyses are required to address these issues.

335 These findings open many important questions for future work. Structural insights into
336 latrophilin activation are needed, as are insights into the contribution of the large extracellular
337 domain. The parallel roles of Gi2 and Gq/11 and downstream effectors remain to be understood.
338 Thorough analysis of latrophilin functions in vascular development and physiology remains to be
339 done. Lastly, combined human genetic and structure-function studies are needed to elucidate the
340 contribution of Lphn2 variants to human cardiovascular disease.

341

342 **Experimental Procedures**

343

344 **Antibodies**

345 Primary antibodies used in this study:

Epitope	Vendor	Catalog #
Phospho-Src	Cell signaling	6943
Src	Cell signaling	2109
Gi	NewEast Biosciences	26003
Gq	NewEast Biosciences	26060
Gs	BD Bioscience	612705
Phospho-VEGFR2(Tyr1175)	NewEast Biosciences	26006
Phospho-VEGFR3 (Tyr1054/Tyr1059)	Cell signaling	2478
VEGFR2	Invitrogen	44-1047G
Phospho-Akt (Ser473)	Cell signaling	2479
VE-Cadherin	Cell signaling	9271
LPHN2	Santa Cruz	sc-6458
RFP	Invitrogen	PA5-65359
alpha-smooth muscle actin	Antibodies-Online	ABIN129578
alpha-smooth muscle actin-Cy3-conjugated	Invitrogen	50-9760-82
ZO-1	Sigma	C6198
beta-catenin	Invitrogen	61-7300
GluGlu	Cell signaling	9562
HA	BioLegend	MMS-115P
GFP	BioLegend	901501
GST	Abcam	ab13970
tubulin	Santa Cruz	sc-9996
actin	Cell signaling	2625
PECAM1	Invitrogen	62204
GM130	Santa Cruz	sc-8432
	Kind gift from Dr. Peter Newman	
	BD Bioscience	557355
	BD Bioscience	610823

346

347

348

349 **Cell Culture**

350 Primary HUVECs were obtained from the Yale Vascular Biology and Therapeutics core
351 facility. Each batch is composed of cells pooled from three donors. Cells were cultured in M199
352 (Gibco: 11150-059) supplemented with 20% FBS, 1x Penicillin-Streptomycin (Gibco: 15140-
353 122), 60 µg/ml heparin (Sigma: H3393), and endothelial growth cell supplement (hereafter,
354 complete medium). HUVECs used for experiments were between passages 3 and 6.

355

356 **Shear stress**

357 For generating protein lysates for pulldown assays, HUVECs were seeded on tissue culture-
358 treated plastic slides coated with 10 µg/ml fibronectin for 1 hour at 37°C and then grown to
359 confluence in HUVEC complete media. For short-term stimulation with shear stress, cells were
360 starved overnight in M199 medium with 2% FBS and 1:10 of ECGS or for 30 minutes in M199
361 medium containing 0.2 % BSA. These slides were set in parallel flow chambers and shear stress
362 applied as described(Frangos *et al*, 1988).

363 For imaging cells under shear stress, HUVECs were plated in HUVEC complete medium on
364 glass bottom 6-well plates coated with 10 µg/ml fibronectin and shear stress applied on an orbital
365 shaker at 150rpm for 24 hours, leading to uniaxial pulsatile shear in the peripheral region and
366 multiaxial shear in the middle of the well, as previously described(Arshad *et al*, 2021). Cells in
367 the outer region, 0.7-0.9 cm from the center of each well were examined for Golgi orientation
368 and endothelial alignment.

369

370 **Image analysis**

371 Cell orientation was calculated by taking the masks of the cell nuclei determined by Hoechst
372 images, fitting them as an ellipse, and determining the angle between flow direction and the
373 major axis of the ellipse. Analyzed results were visualized as histograms showing the percent of
374 cells within each 10° of the direction of flow or as quantification of aligned cells with nuclei
375 whose major axis were within 0-30 degrees to flow direction as indicated in the figure legends.

376 Eccentricity for cell alignment was calculated based on first eccentricity of ellipse following the
377 equation below.

378

$$Eccentricity = \frac{\sqrt{a^2 - b^2}}{a}$$

379 (a: major axis, b:minor axis)

380

381 Junctional linearization index was calculated as ratio of perimeter of the cell to perimeter of
382 its convex hull following the equation below:

383

$$Junctional\ linearization\ index = \frac{exact\ contour\ of\ the\ cell}{convex\ perimeter}$$

384

385 **Data display**

386 Quantified data are displayed as means \pm standard error (SEM) in which the indicated
387 replicates are independent experiments. F test was conducted to check equal variance.

388

389 **Lentiviral transduction**

390 Lenti-X 293T cells (Clontech, 632180) were cultured for at least 24 hours in DMEM
391 supplemented with 10% FBS and lacking antibiotics, then transfected with lentiviral plasmids
392 encoding the gene of interest and packaging plasmids (Addgene: 12259 and 12260) using
393 Lipofectamine 2000 (Thermo Fisher Scientific: 11668-019) following the manufacturer's
394 protocols with Opti-MEM medium. Conditioned media from these cultures were collected 48
395 hours later, sterilized through 0.22 μ m filters and added to HUVECs together with 8 μ g/ml of
396 polybrene (Sigma: 107689). After 24 hours, cells were switched to complete medium for 48
397 hours.

398

399 **siRNA transfection**

400 HUVECs were cultured in EGMTM-2 Endothelial Cell Growth Medium-2 BulletKitTM
401 (Lonza: CC-3156 and CC-4176) for 24 hours before transfecting with RNAiMax (Thermo
402 Fisher Scientific: 13778-150) with 20nM siRNA in Opti-MEM (Gibco: 31985-070) according to
403 the manufacturer's instructions. After 6 hours, cells were switched to EGM-2 medium and used
404 for experiments 2-3 days later. Gα protein siRNAs were custom designed based on previous
405 publications(Grzelinski *et al*, 2010; Krumins & Gilman, 2006; Ngai *et al*, 2008). For latrophilin
406 knockdown, ON-TARGET plus Smartpool siRNAs from Dharmacon against human LPHN1 (L-
407 005650-00-0005), LPHN2 (L-005651-00-0005), LPHN3 (L-005652-00-0005) were used.

408

409 **Western Blotting**

410 HUVECs were washed with PBS and extracted in Laemmli sample buffer. Samples were
411 separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were
412 blocked with 5% milk in TBS-T and probed with primary antibodies at 4°C for overnight. The
413 targeting proteins were visualized by HRP-conjugated secondary antibodies and subsequent
414 HRP-luminol reaction.

415

416 **Immunofluorescence**

417 HUVECs were washed with PBS, fixed for 10 minutes with 3.7% PFA in PBS. Following
418 fixation, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes and then
419 incubated with 3% BSA in PBS for 30 minutes for blocking. Cells were washed with PBS after
420 blocking and were incubated Alexa488-Phalloidin and Hoechst for 1 hour, then washed 4 times
421 with PBS and mounted. Images were captured with 20x or 60x objective on a PerkinElmer
422 spinning disk confocal microscope or a Leica Sp8 confocal microscope with the Leica
423 Application Suite (LAS) software. Cell alignment was determined as described
424 previously(Baeyens *et al*, 2014).

425

426 **Reverse transcription and quantitative PCR**

427 RNA was isolated from HUVECs using RNeasy kit according to the manufacturer's
428 instructions and quantified using a nanodrop spectrophotometer. Following cDNA synthesis
429 using Bio-Rad iScript kit, RT-PCR was performed as follows. Each PCR reaction contains 42
430 two-step amplification cycles consisting of: (a) denaturation at 95°C for 30s, and (b) annealing
431 and extension at 60°C for 30s. The amplification curve was collected, and the relative transcript
432 level of the target mRNA in each sample was calculated by normalization of Ct values to the
433 reference mRNA (GAPDH). Primer sequences used for RT-PCR were as shown in Table 1.

434

435

436 **G_a protein pulldown assay**

437 BL21 *E.coli* cells were transformed with constructs expressing GST-tagged GINIP protein
438 or GST-tagged GRK2 N-terminal domain. Cells were incubated in terrific broth
439 (<http://cshprotocols.cshlp.org/content/2015/9/pdb.rec085894.full>) and protein expression induced
440 by addition of 0.5μg/ml IPTG. Cells were collected after 8 hours, lysed and GST- proteins
441 collected on Glutathione-conjugated beads. Beads were washed 4 times, eluted with 50mM
442 glutathione and proteins desalted on a gel filtration column. Aliquots of 20 μg aliquots were
443 stored frozen and thawed shortly before use.

444 For pulldown assays, HUVECs infected with lentivirus encoding GluGlu-tagged Gi mutant
445 or lentivirus encoding both GluGlu-tagged Gq mutant and Ric8A were lysed in cell lysis buffer
446 composed of 10mM Tris-HCl pH7.5, 150mM NaCl, 1% Triton X-100, 5mM DTT and additional
447 2μg/ml of GST-GINIP or GST-GRK2N, and the lysates were incubated for 10 minutes at 4°C
448 with gentle agitation with glutathione beads. Beads were washed three times with cell lysis
449 buffer, isolated proteins were solubilized in SDS sample laemmli buffer, and were analyzed by
450 western blotting.

451

452

453 **Mass spectrometry screening for G α binding receptors**

454 To create a G α protein variant that interacts more strongly with GPCRs, a green fluorescent
455 protein (GFP) tag was inserted for affinity purification, alongside a mutation of four alanine
456 residues into helix α 5 (ins4A), as this hinders GTP binding and maintains the association with
457 GPCRs even in the presence of GTP. Additionally, for the extraction of GPCRs while
458 preserving their configuration and lipid environment, a non-detergent nanodisc approach
459 utilizing styrene-maleimide anhydride (SMA) copolymers was employed according to protocols
460 described previously.

461 In the coimmunoprecipitation process, GFP-Trap® nanobody beads were utilized to capture
462 the interactions between GFP-tagged G α protein variants and activated designer receptors
463 exclusively activated by designer drugs (DREADDs). The resulting complexes were subjected to
464 subsequent proteomic analysis to determine which GPCRs interacted preferentially with the
465 Gi1(Q306K) variant following induction of fluid shear stress. Identified receptors were further
466 analyzed by western blotting as described in the supplementary material.

467

468

469 **Measurement of Golgi orientation**

470 Following exposure to laminar shear stress, HUVECs were washed with PBS, fixed for 10
471 minutes with 3.7% PFA in PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes,
472 blocked with 3% BSA in PBS for 30 minutes and then incubated with GM130 antibody (1:500,
473 BD bioscience) at 4°C for overnight. After washing, cells were incubated with Hoechst and
474 Alexa647-conjugated secondary antibody at RT for 1 hour to visualize the nuclei and Golgi
475 apparatus, respectively. Images were captured with 20x objective on a Leica Sp8 confocal
476 microscope with the Leica Application Suite (LAS) software.

477 Golgi polarization was calculated as a vector that connects the center of mass of the nucleus
478 to the center of mass of the Golgi. The angle between this vector and the direction of flow was

479 then determined, with 0° defined as against the flow. Oriana V4 software was used to calculate
480 the angle mean and circular SD and to generate a rose plot for data visualization.

481

482 ***In vitro* permeability assay using the FITC-streptavidin/biotinylated-fibronectin**

483 Slides/dishes were coated with biotinylated-Fibronectin at 0.25 mg/mL overnight at 4°C.
484 HUVECs were seeded of 2×10^6 cells per well on glass-bottom 6-well plates.
485 Cells were subjected to laminar shear stress by orbital shaking at 150 rpm for 24
486 hours. Immediately after cessation of flow, FITC-Streptavidin (1:1000, Invitrogen) was added to
487 the cells for 1 minute before the fixation by the addition of 3.7% (v/v) paraformaldehyde.
488 Following subsequent washing and blocking, Alexa Fluor 488 Phalloidin (1:200;
489 Invitrogen) was added to stain F-actin filaments. Images were captured from 5 random fields
490 and FITC-streptavidin staining quantified using ImageJ.

491

492 ***In vitro* endothelial 3D invasion assay under shear stress**

493 In order to equalize the VEGF concentration in the collagen gel and endothelial culture
494 medium, VEGF-189 (R&D systems, 8147-VE), a matrix-binding isoform of VEGF, was applied
495 to the bottom of each well at a concentration of 50 ng/ml prior to adding collagen matrices. Rat
496 tail collagen type I (Thermofisher scientific, A1048301) was employed to prepare collagen
497 matrices at the concentration of 1.5 mg/ml with additional NaOH to facilitate the collagen matrix
498 assembly by neutral pH. After thorough mixing, 1 ml of collagen I solution was placed in each
499 well of a 12-well plate (Corning #3513) and polymerized at 37°C and 5% CO₂ for 2 hours to
500 overnight.

501 To prepare endothelial cells, confluent HUVEC culture plates were washed with PBS,
502 trypsinized, and counted. After pelleting, cell pellets were resuspended in EGM-2 medium
503 (Lonza, #CC-3162) free of VEGF and heparin to establish a positive gradient of VEGF from
504 cells to the bottom of the collagen gel, and 300,000 cells per well were placed on the collagen gel
505 and incubated for 24 hours. Following incubation, plates were placed on an orbital shaker and

506 ECs were exposed to orbital shaking at 150 rpm for 48 hours as mentioned above in shear stress
507 section. Cells were then fixed in 4% PFA and permeabilized in PBS containing 0.5% Triton X-
508 100. Nonspecific binding was blocked by incubation with PBS containing 3% BSA. Thereafter,
509 cells were subjected to Alexa488-conjugated phalloidin (1:400 dilution; Thermofisher scientific:
510 A12379) and Hoechst (1:1000 dilution; Invitrogen: H1399) for 2 hours to stain actin
511 cytoskeleton and cell nuclei, respectively. All images were captured with the Nikon Sp8 confocal
512 microscope and segregated into peripheral region (with uniaxial pulsatile shear) vs. static
513 controls.

514

515 **Zebrafish husbandry and handling**

516 Zebrafish were housed and maintained at 28.5°C in accordance with standard methods
517 approved by the Yale University Institutional Animal Care and Use Committee (#2017-
518 11473)(Lawrence, 2016).

519

520 **Generation of LPHN/adgrl knockdown zebrafish using CRISPR/Cas9 ribonucleoproteins**

521 Four zebrafish latrophilin paralogs were identified—*adgrl1a*, *adgrl2a*, *adgrl2b.1*, and
522 *adgrl3.1*. Fused sgRNAs were generated targeting each paralog (see Table 2) by annealing locus-
523 specific oligonucleotides to a common 5' universal oligonucleotide and performing *in vitro* RNA
524 transcription (AmpliScribe T7-Flash Kit, Lucigen, ASF3507)(Gagnon *et al.*, 2014). An injection
525 mix of 50 ng/µL each sgRNA, an equivalent concentration of Cas9 protein (TrueCut Cas9,
526 Invitrogen, A36497), and either 1 µg/µL *silent heart/tnt2a*(Sehnert *et al.*, 2002) or standard
527 control morpholino (GeneTools) was prepared. 1 nL of the mixture was injected into
528 *Tg(kdrl:ras-mCherry, fli1a:nls-GFP)* zebrafish at the one cell stage (Parker Hannifin,
529 Picospritzer III). CRISPR reagent efficacy was confirmed by T7 endonuclease assay (see Figure
530 S5). Guides against adgrl2a and 2b.1 were combined to target all LPHN2 isoforms.

531

532 **Immunostaining and imaging of ECs in the knockdown zebrafish**

533 CRISPR/Cas9 RNP-injected embryos were either fixed at 48 hpf in 4% paraformaldehyde
534 (Santa Cruz Biotechnology, SC-253236) and immunostained for GFP (1:300 chicken anti-GFP,
535 abcam, ab13970), mCherry (1:300 rabbit anti-mCherry, abcam, ab167453), and ZO-1 (1:200
536 mouse anti-ZO-1, Invitrogen, 61-7300) with species-appropriate secondary antibodies (1:400
537 Invitrogen anti-chicken/-rabbit/-mouse Alexa 488/546/647, A-11039/A- 10040, A-31571) or
538 imaged live. For fixed imaging, the larvae were processed as previously described(Sugden *et al.*,
539 2017). For live imaging, larvae were anesthetized by immersion in 600 µM tricaine
540 methylsulfonate (Western Chemical, TRS5), and embedded in 1.5% low-melt agarose (BioRad,
541 1613106). For staining in detail, larvae were fixed overnight in 4% PFA at +4°C and then
542 transitioned into 100% methanol via a series of dilutions. Once in methanol, the larvae were
543 stored at -20°C overnight, then rehydrated the next day via the same dilution series. Larvae were
544 permeabilized using incubation in acetone for 15 minutes at -20°C and then by incubation in 20
545 µg/mL proteinase K for 20 minutes at 37°C. Larvae were refined in 4% PFA for 15 minutes at
546 room temperature and then incubated in blocking buffer (0.5% Triton X-100, 10% goat serum,
547 1% BSA, 0.01% sodium azide, and 1% DMSO in PBS) for 2 hours at room temperature. Larvae
548 were incubated in primary antibody (1:400 chicken anti-GFP, abcam ab13970) overnight in
549 blocking buffer at +4°C. Larvae were thoroughly washed in PBS-Tx and incubated overnight at
550 +4°C in secondary antibody (1:400 goat anti-chicken Alexa488 A-11039, ThermoFisher) in
551 blocking buffer. Following final washes in PBS-Tx, larvae were mounted and imaged.
552 Intersegmental vessel and dorsal aorta diameter were then calculated using ImageJ. All imaging
553 was performed using a Leica SP8 confocal microscope.

554

555 **Immunostaining and imaging of mouse aorta**

556 Aortae were perfusion-fixed *in situ* with 4% paraformaldehyde (overnight) prior to staining
557 with primary beta-catenin antibody (Cell signaling: 9562) and fluorophore-conjugated secondary
558 antibody (Invitrogen, donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary
559 Antibody, Alexa Fluor 647, A31573) in in Claudio buffer (1% FBS, 3% BSA, 0.5% Triton X-

560 100, 0.01% Sodium deoxycholate, 0.02% sodium azide in PBS pH7.4)(Franco *et al*, 2013).
561 Cells were stained with Hoechst (Invitrogen: H1399) to label nuclei. Images of stained vessels
562 were acquired using a Leica SP8 confocal microscope with the Leica Application Suite (LAS)
563 software. Endothelial cell alignment in aorta was quantified by measuring the eccentricity of the
564 immunostaining of beta-catenin of the endothelial cells (>150 cells per field of view).

565

566 **Extravasation of Evans blue dye for aortic vascular permeability**

567 For aortic vascular permeability measurements, Evans blue dye (20 mg/ml in saline, 2.5 µl/g
568 mouse body weight) (Sigma E2129) was injected retro-orbitally to LPHN2 endothelial cell-
569 specific knockout and their control littermates at 8 weeks old. Thirty minutes post injection,
570 animals were euthanized by an overdose of isoflurane and tissue samples including aorta were
571 harvested and washed with PBS to remove extra fatty tissues. Dissected aorta was prepared *en*
572 *face* and imaged with an Olympus stereo zoom microscope. To quantify blue color associated
573 with extravasation of Evans blue dye, the original RGB images were converted into HSV color
574 model, and the proportion of the blue regions was calculated as the ratio of pixels with hue
575 angles of 120°-240° to the entire aorta.

576

577 **Immunostaining and imaging of mouse muscle capillaries**

578 Rectus femoris muscle from murine thigh were perfusion-fixed with 4% paraformaldehyde
579 (overnight) and the middle part of the muscle were cryo-sectioned following embedding into
580 OCT compound and stained with primary antibodies for SMA (fluorophore-conjugated) and for
581 CD31 followed by fluorophore-conjugated secondary antibody (Invitrogen, donkey anti-Rabbit
582 IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647, A31573) in PBS
583 containing 5% BSA. Hoechst (Invitrogen: H1399) was used to nuclear identification. Images of
584 stained vessels were acquired using a Leica SP8 confocal microscope with the Leica Application
585 Suite (LAS) software.

586

587 **Hindlimb ischemia model**

588 Mice were anesthetized and operated with a mixture of 1.5-2% isoflurane. The front hair of
589 the right thigh was removed with Nair lotion and the local surgical area was disinfected. A 5 mm
590 longitudinal skin incision was made in the right thigh. The distal femoral artery, proximal to the
591 popliteal artery and saphenous artery, was explored and dissected. 6-0 silk sutures (Syneture)
592 were double used for the ligation before excision of the arterial vessel bed between the distal end
593 of the superficial epigastric artery and the trifurcation of the femoral artery into the descending
594 genicular, popliteal, and sphenous branches. The ligated artery was cut between two sutures.
595 The venous structures and accompanying peripheral nerves were kept intact. The overlying skin
596 was closed with a 6-0 prolene (Ethicon) and disinfected. At the end of the procedure,
597 Buprenorphine was administered subcutaneously at 0.05 mg/kg, once pre-emptively, then every
598 12 h for 72 h. The survival rate for this surgery was 100%.

599

600 **Laser Doppler perfusion imaging**

601 Hindlimb perfusion was assessed noninvasively in the plantar foot before, 1, 3, 7, 14, and 21
602 days after hindlimb ischemia by scanning laser-Doppler [model LDI2-IR modified for high
603 resolution; Moor Instruments, Wilmington, DE]. The hindquarters were placed on the top of a
604 heater pad during scanning to minimize variation in body temperature. Doppler perfusion of the
605 plantar foot was assessed within anatomically defined regions of interest (ROIs; Moor
606 Instruments). The ROI for the plantar foot consisted of the hind paw margins. Procedures for
607 minimal preparation, scanning, and ROI selections have been described in detail
608 previously(Zhuang *et al*, 2011). Low or no perfusion is displayed as dark blue, whereas the
609 highest degree of perfusion is displayed as red. These images were quantitatively converted into
610 histograms that represented the amount of blood flow on the x-axis and the number of pixels on
611 the y-axis in the traced area. The average blood flow in each histogram was calculated and the
612 LDI index was determined as the ratio of ischemic to non-ischemic hindlimb blood perfusion.

613

614 **Volumetric micro-CT angiogram for quantitative arteriogenesis**

615 After the mice were euthanized, the vasculature was flushed with 0.9% normal saline
616 containing heparin (1000 IU/L) for 3 minutes, followed by 4% paraformaldehyde for 5 minutes
617 at 100 mm Hg pressure. Fresh home-made 20% bismuth nanoparticles mixed in 5% gelatin were
618 used as a micro-CT contrast agent and injected over 2 minutes with a syringe pump, as described
619 before(Tirziu *et al.*, 2005). The mice were then immediately chilled on ice for more than 30 min
620 and immersion-fixed in 2% paraformaldehyde overnight. The peripheral vasculature in the
621 ischemic hindlimb and the contralateral normal hindlimb was imaged with a high-resolution
622 micro-CT imaging system (GE eXplore Locus SP), set to a 0.007-mm effective detector pixel
623 size. Microview software (GE Healthcare) was used for initial reconstruction. Advanced
624 workstation with various software was used for 3D reconstruction and segmentation of the whole
625 hindlimb at the 21 μm spatial resolution. Using the knee as the breakpoint, we re-sliced the 3D
626 vasculature into 200 slices both in the thigh and in the calf regions. Quantification was
627 performed by use of a NIH Image. The data were expressed as vascular segment numbers,
628 representing the total number of vessels, of specified diameter, counted in 200 z -sections for the
629 thigh region and another 200 z -sections for the calf region.

630

631 **Mouse treadmill fatigue test**

632 Mice were first acclimatized by staying on the motionless treadmill machine for 30 min on
633 day 1 and then trained on the treadmill moving at 12 m/min for 30 min on day 2. Fatigue tests
634 were then conducted on days 3 and 4. Mice were placed on the treadmill and the shock rod was
635 activated. Treadmill speeds were increased as follows: 0 m/min, for 12 min to measure basal
636 metabolism, 12 m/min, for 1 min, then increasing at a rate of +1 m/min each 1 min until
637 exhaustion. Exhaustion (endpoint of treadmill cessation) was defined as the point at which mice
638 continuously contacted with the shock rod for 5 continuous seconds or when mice stopped
639 running 3 times within 5 seconds. Before each testing session, Oxymax software (Columbus
640 Instruments, Columbus, OH, USA) and open circuit indirect calorimetry treadmills (Metabolic

641 Modular Treadmill, Columbus Instruments, Columbus, OH) were calibrated and checked for
642 hardware malfunctions according to manufacturer instructions.

643

644 **Data availability**

645 The datasets produced in this study are available in the following databases:

646 All data: BioStudies S-BIAD928: [https://www.ebi.ac.uk/biostudies/studies?query=S-](https://www.ebi.ac.uk/biostudies/studies?query=S-BIAD928)
647 BIAD928

648

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667

668 **Disclosure & competing interests statement**

669 The authors declare they have no conflict of interest.

670

671

672 **References**

- 673
- 674 Albaran-Juarez J, Iring A, Wang S, Joseph S, Grimm M, Strilic B, Wettschureck N, Althoff TF,
675 Offermanns S (2018) Piezo1 and G(q)/G(11) promote endothelial inflammation depending on
676 flow pattern and integrin activation. *J Exp Med* 215: 2655-2672
- 677 Arshad M, Ghim M, Mohamied Y, Sherwin SJ, Weinberg PD (2021) Endothelial cells do not align
678 with the mean wall shear stress vector. *J R Soc Interface* 18: 20200772
- 679 Bae H, Cabrera-Vera TM, Depree KM, Gruber SG, Hamm HE (1999) Two amino acids within the
680 alpha4 helix of Galphai1 mediate coupling with 5-hydroxytryptamine1B receptors. *J Biol Chem*
681 274: 14963-14971
- 682 Baeyens N, Bandyopadhyay C, Coon BG, Yun S, Schwartz MA (2016) Endothelial fluid shear
683 stress sensing in vascular health and disease. *J Clin Invest* 126: 821-828
- 684 Baeyens N, Mulligan-Kehoe MJ, Corti F, Simon DD, Ross TD, Rhodes JM, Wang TZ, Mejean CO,
685 Simons M, Humphrey J et al (2014) Syndecan 4 is required for endothelial alignment in flow and
686 atheroprotective signaling. *Proc Natl Acad Sci U S A* 111: 17308-17313
- 687 Butler PJ, Norwich G, Weinbaum S, Chien S (2001) Shear stress induces a time- and position-
688 dependent increase in endothelial cell membrane fluidity. *Am J Physiol Cell Physiol* 280: C962-
689 969
- 690 Camillo C, Facchinello N, Villari G, Mana G, Gioelli N, Sandri C, Astone M, Tortarolo D, Clapero F,
691 Gays D et al (2021) LPHN2 inhibits vascular permeability by differential control of endothelial
692 cell adhesion. *J Cell Biol* 220
- 693 Campinho P, Vilfan A, Vermot J (2020) Blood Flow Forces in Shaping the Vascular System: A
694 Focus on Endothelial Cell Behavior. *Front Physiol* 11: 552
- 695 Chachisvilis M, Zhang YL, Frangos JA (2006) G protein-coupled receptors sense fluid shear stress
696 in endothelial cells. *Proc Natl Acad Sci U S A* 103: 15463-15468
- 697 Collins C, Guilluy C, Welch C, O'Brien ET, Hahn K, Superfine R, Burridge K, Tzima E (2012)
698 Localized tensional forces on PECAM-1 elicit a global mechanotransduction response via the
699 integrin-RhoA pathway. *Curr Biol* 22: 2087-2094
- 700 Conway D, Schwartz MA (2012) Lessons from the endothelial junctional mechanosensory
701 complex. *F1000 Biol Rep* 4: 1
- 702 Conway DE, Breckenridge MT, Hinde E, Gratton E, Chen CS, Schwartz MA (2013) Fluid shear
703 stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. *Curr
704 Biol* 23: 1024-1030
- 705 Cornella N, Sancho J, Sitges-Serra A (2017) Short and Long-Term Outcomes After Surgical
706 Procedures Lasting for More Than Six Hours. *Sci Rep* 7: 9221
- 707 Dela Paz NG, Frangos JA (2019) Rapid flow-induced activation of Galphaq/11 is independent of
708 Piezo1 activation. *Am J Physiol Cell Physiol* 316: C741-C752
- 709 Dela Paz NG, Melchior B, Frangos JA (2017) Shear stress induces Galphaq/11 activation
710 independently of G protein-coupled receptor activation in endothelial cells. *Am J Physiol Cell
711 Physiol* 312: C428-C437

- 712 dela Paz NG, Melchior B, Shayo FY, Frangos JA (2014) Heparan sulfates mediate the interaction
713 between platelet endothelial cell adhesion molecule-1 (PECAM-1) and the Galphaq/11 subunits
714 of heterotrimeric G proteins. *J Biol Chem* 289: 7413-7424
- 715 Erdoganmus S, Storch U, Danner L, Becker J, Winter M, Ziegler N, Wirth A, Offermanns S,
716 Hoffmann C, Gudermann T et al (2019) Helix 8 is the essential structural motif of
717 mechanosensitive GPCRs. *Nat Commun* 10: 5784
- 718 Fancher IS, Ahn SJ, Adamos C, Osborn C, Oh MJ, Fang Y, Reardon CA, Getz GS, Phillips SA,
719 Levitan I (2018) Hypercholesterolemia-Induced Loss of Flow-Induced Vasodilation and Lesion
720 Formation in Apolipoprotein E-Deficient Mice Critically Depend on Inwardly Rectifying K(+)
721 Channels. *J Am Heart Assoc* 7
- 722 Flock T, Hauser AS, Lund N, Gloriam DE, Balaji S, Babu MM (2017) Selectivity determinants of
723 GPCR-G-protein binding. *Nature* 545: 317-322
- 724 Franco CA, Blanc J, Parlakian A, Blanco R, Aspalter IM, Kazakova N, Diguet N, Mylonas E, Gao-Li J,
725 Vaahokari A et al (2013) SRF selectively controls tip cell invasive behavior in angiogenesis.
726 *Development* 140: 2321-2333
- 727 Frangos JA, McIntire LV, Eskin SG (1988) Shear stress induced stimulation of mammalian cell
728 metabolism. *Biotechnol Bioeng* 32: 1053-1060
- 729 Gagnon JA, Valen E, Thyme SB, Huang P, Akhmetova L, Pauli A, Montague TG, Zimmerman S,
730 Richter C, Schier AF (2014) Efficient mutagenesis by Cas9 protein-mediated oligonucleotide
731 insertion and large-scale assessment of single-guide RNAs. *PLoS One* 9: e98186
- 732 Gaillard S, Lo Re L, Mantilleri A, Hepp R, Urien L, Malapert P, Alonso S, Deage M, Kambrun C,
733 Landry M et al (2014) GINIP, a Galphai-interacting protein, functions as a key modulator of
734 peripheral GABAB receptor-mediated analgesia. *Neuron* 84: 123-136
- 735 Galie PA, Nguyen DH, Choi CK, Cohen DM, Janmey PA, Chen CS (2014) Fluid shear stress
736 threshold regulates angiogenic sprouting. *Proc Natl Acad Sci U S A* 111: 7968-7973
- 737 Givens C, Tzima E (2016) Endothelial Mechanosignaling: Does One Sensor Fit All? *Antioxid
738 Redox Signal* 25: 373-388
- 739 Grzelinski M, Pinkenburg O, Buch T, Gold M, Stohr S, Kalwa H, Gudermann T, Aigner A (2010)
740 Critical role of G(alpha)12 and G(alpha)13 for human small cell lung cancer cell proliferation in
741 vitro and tumor growth in vivo. *Clin Cancer Res* 16: 1402-1415
- 742 Gudi S, Nolan JP, Frangos JA (1998) Modulation of GTPase activity of G proteins by fluid shear
743 stress and phospholipid composition. *Proc Natl Acad Sci U S A* 95: 2515-2519
- 744 Gudi SR, Clark CB, Frangos JA (1996) Fluid flow rapidly activates G proteins in human
745 endothelial cells. Involvement of G proteins in mechanochemical signal transduction. *Circ Res*
746 79: 834-839
- 747 Haidekker MA, L'Heureux N, Frangos JA (2000) Fluid shear stress increases membrane fluidity in
748 endothelial cells: a study with DCVJ fluorescence. *Am J Physiol Heart Circ Physiol* 278: H1401-
749 1406
- 750 Humphrey JD, Schwartz MA (2021) Vascular Mechanobiology: Homeostasis, Adaptation, and
751 Disease. *Annu Rev Biomed Eng* 23: 1-27
- 752 Jung B, Obinata H, Galvani S, Mendelson K, Ding BS, Skoura A, Kinzel B, Brinkmann V, Rafii S,
753 Evans T et al (2012) Flow-regulated endothelial S1P receptor-1 signaling sustains vascular
754 development. *Dev Cell* 23: 600-610

- 755 Kaya AI, Lokits AD, Gilbert JA, Iverson TM, Meiler J, Hamm HE (2016) A Conserved Hydrophobic
756 Core in Galphai1 Regulates G Protein Activation and Release from Activated Receptor. *J Biol*
757 *Chem* 291: 19674-19686
- 758 Kroon J, Heemskerk N, Kalsbeek MJT, de Waard V, van Rijssel J, van Buul JD (2017) Flow-
759 induced endothelial cell alignment requires the RhoGEF Trio as a scaffold protein to polarize
760 active Rac1 distribution. *Mol Biol Cell* 28: 1745-1753
- 761 Krumins AM, Gilman AG (2006) Targeted knockdown of G protein subunits selectively prevents
762 receptor-mediated modulation of effectors and reveals complex changes in non-targeted
763 signaling proteins. *J Biol Chem* 281: 10250-10262
- 764 Lawrence C (2016) New frontiers for zebrafish management. *Methods Cell Biol* 135: 483-508
- 765 Lee MJ, Evans M, Hla T (1996) The inducible G protein-coupled receptor edg-1 signals via the
766 G(i)/mitogen-activated protein kinase pathway. *J Biol Chem* 271: 11272-11279
- 767 Lee SC, Knowles TJ, Postis VL, Jamshad M, Parslow RA, Lin YP, Goldman A, Sridhar P, Overduin
768 M, Muench SP *et al* (2016) A method for detergent-free isolation of membrane proteins in their
769 local lipid environment. *Nat Protoc* 11: 1149-1162
- 770 Liu SL, Sheng R, Jung JH, Wang L, Stec E, O'Connor MJ, Song S, Bikkavilli RK, Winn RA, Lee D *et al*
771 (2017) Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane
772 cholesterol. *Nat Chem Biol* 13: 268-274
- 773 Lucitti JL, Jones EA, Huang C, Chen J, Fraser SE, Dickinson ME (2007) Vascular remodeling of the
774 mouse yolk sac requires hemodynamic force. *Development* 134: 3317-3326
- 775 Maleszewska M, Vanchin B, Harmsen MC, Krenning G (2016) The decrease in histone
776 methyltransferase EZH2 in response to fluid shear stress alters endothelial gene expression and
777 promotes quiescence. *Angiogenesis* 19: 9-24
- 778 Medina R, Grishina G, Meloni EG, Muth TR, Berlot CH (1996) Localization of the effector-
779 specifying regions of Gi2alpha and Gqalpha. *J Biol Chem* 271: 24720-24727
- 780 Nazarko O, Kibrom A, Winkler J, Leon K, Stoveken H, Salzman G, Merdas K, Lu Y, Narkhede P,
781 Tall G *et al* (2018) A Comprehensive Mutagenesis Screen of the Adhesion GPCR Latrophilin-
782 1/ADGRL1. *iScience* 3: 264-278
- 783 Ngai J, Methi T, Andressen KW, Levy FO, Torgersen KM, Vang T, Wettschureck N, Tasken K
784 (2008) The heterotrimeric G-protein alpha-subunit Galphaq regulates TCR-mediated immune
785 responses through an Lck-dependent pathway. *Eur J Immunol* 38: 3208-3218
- 786 Papasergi MM, Patel BR, Tall GG (2015) The G protein alpha chaperone Ric-8 as a potential
787 therapeutic target. *Mol Pharmacol* 87: 52-63
- 788 Privratsky JR, Newman DK, Newman PJ (2010) PECAM-1: conflicts of interest in inflammation.
789 *Life Sci* 87: 69-82
- 790 Sando R, Jiang X, Sudhof TC (2019) Latrophilin GPCRs direct synapse specificity by coincident
791 binding of FLRTs and teneurins. *Science* 363
- 792 Sehnert AJ, Huq A, Weinstein BM, Walker C, Fishman M, Stainier DY (2002) Cardiac troponin T is
793 essential in sarcomere assembly and cardiac contractility. *Nat Genet* 31: 106-110
- 794 Strohbach A, Pennewitz M, Glaubitz M, Palankar R, Gross S, Lorenz F, Materzok I, Rong A, Busch
795 MC, Felix SB *et al* (2018) The apelin receptor influences biomechanical and morphological
796 properties of endothelial cells. *J Cell Physiol* 233: 6250-6261

- 797 Sugden WW, Meissner R, Aegeater-Wilmsen T, Tsaryk R, Leonard EV, Bussmann J, Hamm MJ,
798 Herzog W, Jin Y, Jakobsson L *et al* (2017) Endoglin controls blood vessel diameter through
799 endothelial cell shape changes in response to haemodynamic cues. *Nat Cell Biol* 19: 653-665
800 Tesmer VM, Kawano T, Shankaranarayanan A, Kozasa T, Tesmer JJ (2005) Snapshot of activated
801 G proteins at the membrane: the Galphaq-GRK2-Gbetagamma complex. *Science* 310: 1686-
802 1690
803 Tirziu D, Moodie KL, Zhuang ZW, Singer K, Helisch A, Dunn JF, Li W, Singh J, Simons M (2005)
804 Delayed arteriogenesis in hypercholesterolemic mice. *Circulation* 112: 2501-2509
805 Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, Cao G, DeLisser H,
806 Schwartz MA (2005) A mechanosensory complex that mediates the endothelial cell response to
807 fluid shear stress. *Nature* 437: 426-431
808 Vizurraga A, Adhikari R, Yeung J, Yu M, Tall GG (2020) Mechanisms of adhesion G protein-
809 coupled receptor activation. *J Biol Chem* 295: 14065-14083
810 Vogel J, Niederer D, Jung G, Troidl K (2020) Exercise-Induced Vascular Adaptations under
811 Artificially Versus Pathologically Reduced Blood Flow: A Focus Review with Special Emphasis on
812 Arteriogenesis. *Cells* 9
813 Wang S, Iring A, Strilic B, Albaran Juarez J, Kaur H, Troidl K, Tonack S, Burbiel JC, Muller CE,
814 Fleming I *et al* (2015) P2Y(2) and Gq/G(1)(1) control blood pressure by mediating endothelial
815 mechanotransduction. *J Clin Invest* 125: 3077-3086
816 Wilson PT, Bourne HR (1995) Fatty acylation of alpha z. Effects of palmitoylation and
817 myristoylation on alpha z signaling. *J Biol Chem* 270: 9667-9675
818 Xu J, Mathur J, Vessieres E, Hammack S, Nonomura K, Favre J, Grimaud L, Petrus M, Francisco A,
819 Li J *et al* (2018) GPR68 Senses Flow and Is Essential for Vascular Physiology. *Cell* 173: 762-775
820 e716
821 Yamamoto K, Ando J (2013) Endothelial cell and model membranes respond to shear stress by
822 rapidly decreasing the order of their lipid phases. *J Cell Sci* 126: 1227-1234
823 Yamamoto K, Ando J (2015) Vascular endothelial cell membranes differentiate between stretch
824 and shear stress through transitions in their lipid phases. *Am J Physiol Heart Circ Physiol* 309:
825 H1178-1185
826 Yamamoto K, Nogimori Y, Imamura H, Ando J (2020) Shear stress activates mitochondrial
827 oxidative phosphorylation by reducing plasma membrane cholesterol in vascular endothelial
828 cells. *Proc Natl Acad Sci U S A* 117: 33660-33667
829 Yeh JC, Otte LA, Frangos JA (2008) Regulation of G protein-coupled receptor activities by the
830 platelet-endothelial cell adhesion molecule, PECAM-1. *Biochemistry* 47: 9029-9039
831 Zhou J, Li YS, Chien S (2014) Shear stress-initiated signaling and its regulation of endothelial
832 function. *Arterioscler Thromb Vasc Biol* 34: 2191-2198
833 Zhuang ZW, Shi J, Rhodes JM, Tsapakos MJ, Simons M (2011) Challenging the surgical rodent
834 hindlimb ischemia model with the miniinterventional technique. *J Vasc Interv Radiol* 22: 1437-
835 1446
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838 **Figure 1. G α proteins specific for endothelial flow responses**

839 **A**, Flow-induced endothelial alignment after G α knockdown. HUVECs were subjected to fluid
840 shear stress (FSS) at a rate of 12 dynes/cm² for 16 hours and nuclear orientation quantified as
841 histograms showing the percentage of cells within each 10° of the flow directions from 0° to 90°
842 (see Methods) ****: p<0.0001; one-way ANOVA with Tukey's multiple comparisons test. **B**,
843 Src family kinase activation, quantified in **C**. n=5 for control, Gi knockdown, Gq11 knockdown
844 and n=3 for simultaneous knockdown of Gi and Gq11. Values are means ± SEM. ****:
845 p<0.0001, ***: p<0.001; one-way ANOVA with Tukey multiple comparison test. **D**, Rescue of
846 Gq/11 and Gi knockdown by re-expression of siRNA-resistant versions of the indicated proteins.
847 ****: p<0.0001; one-way ANOVA with Tukey's multiple comparison test. **E**, Amino acid
848 sequences of G α i1, G α i2, and G α i3 at the mutation sites of G α i1 gain-of-function mutant. **F**,
849 Rescue of Gq/11 and Gi knockdown with indicated G α proteins. Each point corresponds to one
850 measurement averaged from >500 cells. N=4. ****: p<0.0001; one-way ANOVA with Tukey's
851 multiple comparisons test. **G**, GINIP pulldown assay for activation of G α i2 by FSS. N=3.
852 Results quantified in **H**. **: p=0.0185, *Student's t-test*. **I**, GRK2N pulldown assay for activation
853 of Gq. N=4, quantified in **J**. *: p<0.05, *Student's t-test*. **K**, GINIP pulldown assay for FSS-
854 induced activation of wild type and Q306K G α i1. N = 4, quantified in **L**. *: p=0.0304, ***:
855 p=0.0017; *Student's t-test*. **M**, Gi2 activation after PECAM-1 knockdown. HUVECs expressing
856 GluGlu tagged Gi2 were transfected with scrambled siRNA or PECAM-1 siRNA, exposed to
857 FSS, and Gi2 activation assayed as described above, quantified in **N**. Values are means ± SEM,
858 normalized to input G α protein levels. ***: p<0.001; *Student's t-test*, N=4.

859

860 **Figure 2. Identification of the upstream flow-responsive GPCR**

861 **A**, Strategy for identification of GPCRs that bind the gain-of-function Gi1Q306K mutant but not
862 the wild type Gi1. **B**, Co-immunoprecipitation of Gi1Q306K(ins4A) with endogenous LPHN2
863 with and without FSS for 2 min. N=3, quantified in **C**. *: p=0.0374, *Student's t-test*. **D**, Co-
864 immunoprecipitation of LPHN2-GFP with the indicated G α proteins containing internal GluGlu

865 epitope tags. **E**, Gi2 pulldown assay after LPHN2 knockdown. N= 4. Results quantified in **F**.
866 ***: p<0.001, *Student's t-test*.

867

868 **Figure 3. Latrophilins regulate endothelial flow responses**

869 **A**, HUVECs with or without latrophilin-2 knockdown were subjected to FSS for 16 h, then fixed
870 and stained with Hoechst (nuclei), phalloidin (F-actin) and an antibody against VE-Cadherin.
871 Scale bar: 100μm. **B**, Alignment of HUVECs (each bar = 10° increments) after knockdown of
872 latrophilin isoforms was quantified as in Fig. 1 from >2000 cells/experiment, N=3. ****:
873 p<0.0001; one-way ANOVA with Tukey's multiple comparisons test. **C**, Localization of
874 LPHN2-mClover3. Scale bar: 100μm. N=6. **D**, Gi1(Q306K) pulldown from ECs ± FSS for 5 min
875 or for 24 hours, probed for PECAM-1. N=3, quantified in **E**. **F**, Gi1(Q306K) pulldown from ECs
876 ± FSS for 5 min or for 24 hours probed for VE-cadherin. N=3, quantified in **G**. **H**, Activation of
877 Src family kinases and Akt by FSS in HUVECs depleted for latrophilin isoforms. N=3-4,
878 quantified in **I** & **J**. *: p=0.0184, **: p<0.001; one-way ANOVA with Tukey's multiple
879 comparisons test. **K**, Alignment of LPHN2-depleted HUVECs rescued by re-expression of the
880 indicated latrophilin isoforms. ****: p<0.0001; one-way ANOVA with Tukey's multiple
881 comparisons test. Quantification of >2000 cells for each condition, N=3. **L**, LPHN2 knockdown
882 HUVECs were rescued by re-expression of the indicated LPHN2 mutants. ****: p<0.0001, one-
883 way ANOVA with Tukey's multiple comparisons test. Quantification of >500 cells for each
884 condition.

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886 **Figure 4. Latrophilins in flow-mediated endothelial cell morphology *in vivo***

887 **A**. Representative image of HUVECs transfected with scrambled siRNA or LPHN2 siRNA.
888 Outlines of ECs from VE-cadherin staining are shown to next to each image. Scale Bar: 100μm.
889 **B**, Quantification of junctional linearization index from **A**. n=30 for each condition. **:
890 p=0.0019, Statistics is done using two-way ANOVA with Sidak's multiple comparison test. **C**,
891 Dorsal aortas from 48hpf Tg(*kdrl:ras-mCherry*) WT or LPHN mutant zebrafish embryos stained

for ZO-1. EC were outlined as in Methods. LPHN2 mutant indicates *adgrl2a+adgrl2b.1* sgRNAs. **D&E**, Quantification of cell size and eccentricity in embryos with latrophilin-2 CRISPR sgRNAs ± *silent heart* morpholinos (MO). N=6 per condition. ****: p<0.0001, *: p=0.0267, **: p=0.0015, ***: p=0.0008; one-way ANOVA with Tukey's multiple comparisons test. **F**, ECs in the thoracic aorta in 18 weeks old mice at 10 weeks after tamoxifen injections, stained for β-catenin (left) and outlined (right). Data are representative of 6 mice for each condition. Scale Bar: 100μm. **G&H**, quantification of cell eccentricity and junction linearity in mouse aorta. N=4 per condition. **: p=0.0013; ****: p<0.0001; *Student's t-test*. **I**, Aortas from mice 30 min after Evans blue dye injection, thresholded images on the right. Fractional blue area quantified in **J**. n=3 for each condition. *: p=0.0419; *Student's t-test*. **K**, *in vitro* endothelial permeability assay using FITC-streptavidin and biotin-conjugated fibronectin. Scale Bar: 100μm. **L**, Permeability was quantified by measuring FITC-streptavidin area per image field from **K**. n=5, *: p=0.0457, one-way ANOVA.

Figure 5. Cholesterol depletion activates Latrophilin-dependent signaling
A, ECs were treated with FSS or 5 mM methyl-β-cyclodextrin (mβCD) for 1 min then incubated with D4H-mClover3, rinsed and bound mClover3 quantified as described in Methods. N=27 for each condition. ****: p<0.0001; One-way ANOVA with Tukey multiple comparisons test. **B**, Phalloidin staining following short acute treatment of methyl-β-cyclodextrin (mβCD). Scale bar: 100μm. **C**, Co-immunoprecipitation of Gi1Q306K(ins4A) with endogenous Latrophilin-2 (LPHN2) after 5mM MβCD for 1min. **D**, Activation of Src family kinases and VEGFR2 after 5mM MβCD for 1min in HUVECs ± LPHN2 knockdown. Results quantified in **E** and **F**, respectively. Values are means ± SEM. N=5. *: p=0.0305, ***: p=0.0002; one-way ANOVA with Tukey multiple comparison test. **G**, Model for junctional endothelial shear stress mechanotransduction.

Figure 6. Latrophilin-2 in flow-induced vascular remodeling

919 **A**, Representative images of ISVs from 48 hpf embryos with F(0)/mosaic depletions of
920 latrophilin genes or controls. **B**, Quantification of diameters of all ISVs in **A**. n=15 per condition.
921 *: p=0.0452, **: p=0.0150; one-way ANOVA with Tukey's multiple comparisons test. **C**,
922 Quantification of diameters of arterial and venous ISVs at 72 hpf of zebrafish. n=6 per condition.
923 **: p=0.0013; *Student's t-test*. **D**, Ratio of blood flow in the right ischemic vs left control foot by
924 laser doppler, quantified in **E**. N=7-8 per condition. *#: p=0.0439, *: p=0.0135, **: p=0.0028,
925 ##: p=0.0056, #: p=0.0163; *Student's t-test*. **F**, Micro-CT of arterial vasculature after surgery.
926 Representative reconstructed micro-CT images from LPHN2 ECKO and WT littermates on day
927 22 after surgery, quantified in **G**. n=8 per condition. ****: p<0.0001, ***: p=0.0007, *:
928 p=0.0167; two-way ANOVA with Tukey's multiple comparisons test. **H**, Rectus femoris muscle
929 from thighs of 10 weeks old mice of LPHN2 ECKO or wild-type littermates, stained for CD31
930 and SMA to visualize the entire vasculature and arteries. Scale Bar: 100 μ m. **I**, Quantification of
931 capillary density in the entire stitched thigh muscle images. n=3 for control and n=6 for Lphn2
932 ECKO. ****: p<0.0001; *Student's t-test*. **J**, Schematic of mouse treadmill fatigue test. **K**,
933 Maximum running during test. n=4 for control and n=5 for Lphn2 ECKO. **: p=0.0082;
934 *Student's t-test*. **L**, Running distance for each mouse. n=4 for control and n=5 for Lphn2 ECKO.
935 **: p=0.0064; *Student's t-test*. **M**, Oxygen consumption during treadmill fatigue test. *:
936 p<0.0001; two-way ANOVA with Tukey's multiple comparisons test. Control: N=5, LPHN2
937 ECKO: N=4. **N**, Representative 3D reconstructed images at 20° downward angle of ECs plated
938 on top of collagen gels and sprouting under static or flow conditions. ECs were stained with
939 phalloidin; pseudocolors indicate distance from the gel surface as per the color scale on the right.
940 **O**, Histograms of cell area vs. distance from gel surface in **N**. **P**, Mean sprout length. N=4. ***:
941 p=0.0006; one-way ANOVA with Tukey's multi comparison test.

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Table 1: Primer sequences used for quantitative PCR in this study

Gene name	Forward (5'-3')	Reverse (5'-3')
Human LPHN1	CAGTACGACTGTGTCCCCCTACA	GCACCATGCGCCAGACTG
Human LPHN2	GTCCAATATGAATGTGTCCCTTACA	GCACCAAGCACCCGCCTT
Human LPHN3	GTGCAGTATGAATGTGTCCCTTACA	GCACCACGCCAGATTG
Human KLF2	CGGCAAGACCTACACCAAGA	TGGTAGGGCTTCTCACCTGT
Human GAPDH	TGCACCAACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
zebrafish LPHN1a	CAGTATGAATGTGTGCCATACA	ACACCATGCCCGACTG
zebrafish LPHN2a	GTCCAGTATGAATGTGTTCCCTACA	GCACCACGACCCCGCTTG
zebrafish LPHN2b.1	GTCCAGTATGAGTGTGTGCCATACA	TTGCACCAAGCTCCACTC
zebrafish LPHN3.1	GTTCAGTATGAGTGTGTGCCATACA	ACACCATGATCCAGCCTG
zebrafish beta-actin	GATCTGGCATCACACCTTCTAC	TCTTCTCTGTTGGCTTG

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Table 2: Sequences of sgRNAs and morpholinos used in this study

Sequence	Target	Description
5' GCTGTACGAGTATGCTTCATGGG	<i>adgrl1a</i>	sgRNA targeting adgrl1a exon 4
5' CGACGTATAAACTACCCCATCGG	<i>adgrl2a</i>	sgRNA targeting adgrl2a exon 4
5' CCGTCTATGATAAACGCTCCGCC	<i>adgrl2b.1</i>	sgRNA targeting adgrl2b.1 exon 4
5' AGTCCACGGCTGCGATGTATTGG	<i>adgrl3.1</i>	sgRNA targeting adgrl3.1 exon 4
5' CATGTTGCTCTGATCTGACACGCA	<i>tnnt2a</i>	morpholino targeting tnnt2a translation
5' CCTCTTACCTCAGTTACAATTATA	none	control morpholino

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