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Activated I-BAR IRSp53 clustering controls the formation of VASP-actin-based membrane protrusions

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► To cite this version:

Feng-Ching Tsai, J. Michael Henderson, Zack Jarin, Elena Kremneva, Yosuke Senju, et al.. Activated I-BAR IRSp53 clustering controls the formation of VASP-actin-based membrane protrusions. *Science Advances* , 2022, 8 (41), 10.1126/sciadv.abp8677 . hal-03869467

HAL Id: hal-03869467

<https://hal.science/hal-03869467>

Submitted on 24 Nov 2022

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1 **FRONT MATTER**

2

3 **Title**

- 4 • *Full title*

5 Activated I-BAR IRSp53 clustering controls the formation of VASP-actin-based
6 membrane protrusions

- 7 • *Short title*

8 Cell protrusion control by IRSp53

9

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35

36 **Abstract**

37 Filopodia are actin-rich membrane protrusions essential for cell morphogenesis, motility, and
38 cancer invasion. How cells control filopodia initiation on the plasma membrane remains elusive.
39 We performed experiments in cellulo, in vitro and in silico to unravel the mechanism of filopodia
40 initiation driven by the membrane curvature sensor IRSp53. We showed that full-length IRSp53
41 self-assembles into clusters on membranes depending on PIP₂. Using well-controlled in vitro
42 reconstitution systems, we demonstrated that IRSp53 clusters recruit the actin polymerase VASP
43 to assemble actin filaments locally on membranes, leading to the generation of actin-filled
44 membrane protrusions reminiscent of filopodia. By pulling membrane nanotubes from live cells,
45 we observed that IRSp53 can only be enriched and trigger actin assembly in nanotubes at highly
46 dynamic membrane regions. Our work supports a regulation mechanism of IRSp53 in its
47 attributes of curvature sensation and partner recruitment to ensure a precise spatial-temporal
48 control of filopodia initiation.

49

50

51 **Teaser**

52 Curvature sensing protein IRSp53 synergizes with actin elongator VASP to control actin-
53 filled membrane protrusion formation.

54

55 **MAIN TEXT**

56

57 **Introduction**

58 Plasma membrane shaping relies on a precisely controlled coupling of the plasma membrane and
59 the actin cytoskeleton (1-3). A prominent example are filopodia: thin, finger-like membrane
60 protrusions with a typical diameter of 100-300 nm which can extend from cell edges with lengths
61 of the order of 10 μm (4, 5). Filopodia are filled with parallel actin filaments bundled by fascin
62 (typically 10–30 filaments) (6, 7). Cells use filopodia to explore and sense their environment (8).
63 Thus, filopodia are critical in numerous cellular processes, including polarized cell migration and
64 adhesion, and in the tissue environment, embryogenesis, cancer invasion and cell-cell
65 communication (2, 4, 5). Filopodia formation employs different mechanisms involving different
66 sets of actin regulatory proteins (4, 5, 9). So far, two distinct mechanisms of filopodia generation
67 have been proposed: the convergent elongation model relying on the reorganization of the pre-
68 existing Arp2/3 complex-mediated branched actin network in lamellipodia (10-12), and the tip
69 nucleation model in which de novo actin assembly is triggered by formin-family actin nucleators
70 (4). The two mechanisms are not necessarily mutually exclusive and are most likely cell type
71 dependent (4, 13-15). Moreover, it is likely that other distinct mechanisms remained to be
72 unraveled (13). Notably, recent cell biology studies proposed another filopodia generation
73 mechanism in which the membrane curvature sensing protein IRSp53 (insulin receptor substrate
74 p53) forms local clusters on the plasma membrane to recruit the actin polymerase VASP
75 (vasodilator-stimulated phosphoprotein), which promotes actin filament elongation at the onset
76 of filopodia initiation (16-18). Yet, it remains poorly understood how cells control precisely
77 when and where to trigger actin assembly on the plasma membrane to initiate filopodia (4, 5, 9).

78 IRSp53 (also known as BAIAP2), an inverse BAR (I-BAR, Bin-Amphiphysin-
79 Rvs161/167) domain protein, is a crucial player in coordinating actin assembly and membrane
80 dynamics in filopodia formation (16-23). IRSp53 features an N-terminal I-BAR domain (also
81 known as IMD, IRSp53-MIM homology Domain), followed by a CRIB-PR (Cdc42/Rac

82 interactive binding-proline rich) domain, and a canonical SH3 (Src homology 3) domain.
83 Purified I-BAR domains spontaneously assemble into crescent-shaped dimers (23) that can bind
84 to negatively charged lipids such as PS and PI(4,5)P₂ (hereafter as PIP₂) (24, 25). The SH3
85 domain of IRSp53 allows it to interact with many actin regulators, such as VASP (16, 17), Eps8
86 (20), N-WASP (17), WAVE2 (26, 27), and mDia1 (27). Importantly, it was shown that IRSp53
87 exhibits a closed, autoinhibited conformation due to the binding of its SH3 domain to its CRIB-
88 PR motif (28). The most well-known activator of IRSp53 is Cdc42 (16, 17, 19, 28). Additionally,
89 PIP₂ and cytoskeleton effectors, such as Eps8 and VASP, have been shown to synergize with
90 Cdc42 in IRSp53 activation to its fully open conformation (19, 21, 28-30). Importantly, it has
91 been shown that 14-3-3 binds to phosphorylated IRSp53, keeping it in the inhibited state (21, 29,
92 30).

93 Biophysical studies in cellulo and in reconstituted systems have revealed that the I-BAR
94 domain of IRSp53 can sense and generate similar negative membrane curvature that is found in
95 filopodia (25, 31-33). Consistently, when overexpressing either the I-BAR domain or the full-
96 length IRSp53 protein in cells, the generation of membrane protrusions reminiscent of filopodia
97 were observed (16-22). The majority of these I-BAR-induced protrusions contain actin filaments,
98 albeit many of them have a low amount of actin (18). Notably, it was shown that full-length
99 IRSp53 forms clusters on the plasma membrane that are followed by the accumulation of VASP
100 for filopodial initiation (16). The tendency of IRSp53 to cluster was suggested by an in vitro
101 study where, by pulling membrane tubes from giant unilamellar vesicles (GUVs) encapsulating
102 the I-BAR domain, it was shown that at low concentrations and low curvature, the I-BAR
103 domain phase separates along the tubes (32). By using MEF cells derived from IRSp53 null mice
104 stably infected with vectors expressing IRSp53 to levels comparable to the endogenous protein,
105 it was shown that IRSp53 is required for the recruitment and further clustering of VASP at the
106 plasma membrane (16). Moreover, theoretical modeling has predicted how IRSp53 and actin
107 cooperatively drive the formation of membrane protrusions (34). Yet, little is known about how
108 IRSp53 by itself clusters on the plasma membrane, and recruits VASP and actin. Moreover, we
109 still lack a comprehensive mechanistic description of how IRSp53 and VASP cooperate with
110 actin and its regulatory proteins to initiate filopodia.

111 VASP is a member of the Ena/VASP protein family that has been indicated to contribute
112 to filopodia formation in cells (4, 5, 9, 10, 15, 35). VASP forms homo-tetramers that are weakly
113 processive as actin polymerases (36). When forming oligomers or clusters, or when the actin
114 filaments are bundled by fascin, VASP becomes more processive on the barbed ends of the actin
115 filaments (37-40). Consistent with its actin polymerase activity, VASP clusters are part of the
116 filopodial “tip complex”, where actin assembly occurs on the plasma membrane to elongate the
117 filopodium (10, 12, 41). Recent cell biology studies have identified protein partners involved in
118 VASP clustering on the plasma membrane, notably IRSp53, lamellipodin and myosin-X (16, 42,
119 43). While IRSp53-VASP clustering has been proposed to contribute to filopodia formation,
120 lamellipodin and myosin-X-dependent VASP clustering appears to be responsible for the
121 formation of another distinct type of protrusion called microspikes (16, 19, 22, 38, 42-44). Yet,
122 how VASP clusters facilitate filopodial initiation remains to be explored (4, 5).

123 To uncover how IRSp53, VASP and actin cooperate to initiate filopodia, we performed
124 experiments in cellulo, in vitro, and in silico. Using in vitro reconstitution systems, we
125 demonstrated that on PIP₂-membranes, purified full-length IRSp53 self-assembles into clusters
126 that are crucial for the recruitment of VASP to locally trigger actin assembly, giving rise to

127 filopodia-like membrane protrusions. Our coarse-grained simulations showed that PIP₂ is key for
128 IRSp53 clustering. Our in vitro assays revealed that fascin is not required for filopodia initiation;
129 however, we observed that in live cells, fascin enhances filopodia elongation and stability.
130 Finally, to unravel the regulation of IRSp53 activity in live cells, we performed an in cellulo
131 biophysical assay in which membrane nanotubes having a membrane geometry reminiscent of
132 cellular filopodia, but initially devoid of actin, are pulled from the cells using optical tweezers.
133 This assay allows us to reveal the regulated activation of IRSp53 in cells by detecting two of its
134 functions: its ability to sense the negative membrane curvature of the pulled nanotubes and to
135 trigger actin assembly inside the nanotubes. We found that IRSp53 is active only in membrane
136 nanotubes that are pulled from regions of cell surfaces where the plasma membrane exhibits
137 dynamic shape changes. Taken together, our results provide fundamental insights into how the
138 curvature sensor protein IRSp53 synergizes with the actin polymerase VASP in filopodia
139 initiation. Our findings further suggest that IRSp53 is tightly regulated in cells to have a rigorous
140 control over filopodia initiation.

141

142 **Results**

143 **IRSp53 self-assembles into clusters that recruit VASP on PIP₂-membranes**

144 Earlier work on MEF cells derived from IRSp53 null-mice provided evidence that IRSp53 is
145 responsible for the formation of VASP clusters at the leading edge of migrating cells (16). It was
146 shown that the accumulation of IRSp53 precedes the recruitment of VASP prior to the formation
147 of filopodia (16). To further examine the roles of IRSp53 and VASP in filopodia initiation, we
148 expressed IRSp53-eGFP and RFP-VASP in live Rat2 cells, which assemble frequent endogenous
149 filopodia (45). By tracking their fluorescence signals at the plasma membrane, we found that
150 IRSp53 is present along filopodia (Fig. 1A, brackets), while VASP tends to enrich at the
151 filopodial tips (Fig. 1A, arrows). Importantly, before the appearance of filopodia, IRSp53 is
152 enriched at the plasma membrane often as small clusters, while VASP displays quite a diffuse
153 cytoplasmic localization with enrichment at focal adhesions (Fig. 1A). At the onset of a
154 filopodium, the emergence of larger IRSp53 clusters occurs, and this is followed by the birth of
155 VASP clusters (Fig. 1B and Movie S1). To reveal the evolution of IRSp53 and VASP clusters at
156 the plasma membrane before and after filopodia formation, we generated adaptive kymographs
157 with a previously developed automated method (42). The adaptive kymographs follow the
158 movement of the plasma membrane (Fig. 1C) and map the signals of IRSp53 and VASP into
159 vertical lines to create space-time plots (Fig. 1D). The kymographs confirm that IRSp53
160 clustering at the plasma membrane precedes VASP accumulation and filopodia initiation (Fig.
161 1D). To quantify the colocalization of IRSp53-eGFP and RFP-VASP during filopodia formation,
162 we used an intensity correlation analysis by following a well-established method (46) (see
163 Materials and Methods for details). This method allows us to obtain, for each pixel, the
164 normalized mean deviation product of fluorescent signals of IRSp53 and VASP, $(I_{IRSp53} -$
165 $\overline{I_{IRSp53}})(I_{VASP} - \overline{I_{VASP}})/((I_{IRSp53}^{max} - \overline{I_{IRSp53}})(I_{VASP}^{max} - \overline{I_{VASP}}))$, termed the colocalization index,
166 where I , \overline{I} and I^{max} are the intensity of a corresponding pixel, and the mean and the maximum
167 intensities of the image, respectively. For each pixel, the colocalization index indicates how the
168 two protein signals are correlated in terms of their fluorescence intensities. The index varies from
169 -1 to 1 with negative values for non-correlated pixels while positive values for correlated ones.
170 By using this method, we found that the colocalization indexes of IRSp53 and VASP were
171 generally higher than 0, thus indicating their colocalization during filopodia formation (N =28
172 filopodia, n = 2 experiments) (Fig. S1, A and B). Moreover, in 46% of the tracked filopodia, we

173 observed that the values of the IRSp53-VASP colocalization indexes globally increased during
174 the onset of filopodia formation (Fig. S1, C and D). Our results support the earlier work by
175 Disanza et al. (16) and indicate the potential synergistic function of IRSp53 and VASP in
176 filopodia initiation. Our data, together with earlier work (16-18), provide evidence supporting a
177 previously proposed mechanism in which IRSp53 clustering at the plasma membrane induces
178 VASP recruitment to locally polymerize actin on the membrane for driving protrusion formation
179 (16-18). In this scenario, the synergistic functions of IRSp53, VASP, actin, and the filopodia-
180 specific actin filament bundling protein, fascin, would be sufficient to drive protrusion
181 formation.

182 To test this hypothesis in a better-controlled experimental environment than in live cells,
183 we developed an in vitro reconstitution system composed of giant unilamellar vesicles (GUVs)
184 as model membranes, and purified IRSp53, VASP, actin and fascin, all in full-length forms. We
185 first assessed if IRSp53 can self-assemble into clusters on model membranes without the
186 presence of other proteins. We purified full-length human IRSp53 that is a constitutive dimer
187 (23), and labelled it with AX488 dyes (AX488-IRSp53) for visualization by fluorescent confocal
188 microscopy. We generated GUVs containing 5 mole% of PIP₂, given that it is the key
189 phosphatidylinositol for an IRSp53-membrane interaction and thus necessary for filopodia
190 generation (25). We used IRSp53 at 16 nM in its dimer form (i.e., 32 nM as a monomer) to be
191 comparable to the cellular concentration of IRSp53 (29.7 nM to 453 nM, in which 29.7 nM is
192 sufficient for filopodia generation) (17). Interestingly, by incubating AX488-IRSp53 with PIP₂-
193 GUVs, we observed clusters of IRSp53 on the GUV membranes (72%, N = 58 GUVs (technique
194 replicates), n = 3 independent sample preparations (biological replicates) (47)) (Fig. 2A, arrows).
195 These clusters are reminiscent of IRSp53 clusters observed in cells at the onset of filopodia
196 formation. Additionally, we observed that a small fraction of GUVs (usually less than 10% of the
197 whole GUV population) have inward membrane protrusions, where IRSp53 is in the interior of
198 the protrusions (Fig. 2A arrowhead, Fig. S2). This observation demonstrates that the full-length
199 IRSp53 is functional such that it can generate negative membrane curvature.

200 To explore the role of PIP₂ in IRSp53 clustering, we tested if IRSp53 could form clusters
201 upon interacting with another negatively charged lipid, PS, given that IRSp53-PIP₂ binding is
202 driven by electrostatic interactions (25). We replaced PIP₂ with PS (25 mole%, PS has a net
203 charge of -1) on GUVs while keeping the amount of negative charges comparable to that of PIP₂-
204 GUVs (5 mole% of PIP₂. At pH 7, the charge of PIP₂ is expected to be around -4 (48)). We
205 found that IRSp53 can form clusters also on PS-GUVs (Fig. 2B). The number of clusters per
206 GUV are comparable on PIP₂-GUVs and PS-GUVs (Fig. S3). However, the clusters are larger on
207 PIP₂-GUVs as compared to PS-GUVs (Fig. 2C). To elucidate the mechanism underlying
208 IRSp53-clustering on PIP₂-membranes, we performed coarse-grained (CG) simulations. We
209 generated PS-like and PIP₂-like membrane sheets. The PS-like membrane is a quasi-monolayer
210 of membrane beads that uniformly interacts with the I-BAR domain membrane binding surface.
211 The PIP₂-like membranes are nearly identical except a subset of membrane beads (2% or 5%)
212 preferably interact with the ends of the I-BAR domains, as previously reported (25). In both
213 membrane cases, the I-BAR domains have purely repulsive direct interactions with each other
214 and are attracted to each other due to curvature coupling and Casimir-like forces mediated by the
215 membrane as well as a membrane-composition-mediated force that occurs only as PIP₂-like
216 membrane beads cluster to the ends of the I-BAR domains (25). Thus, the functional difference
217 between the PS-like membrane (i.e., 0% PIP₂-like membrane) and the PIP₂-like membrane is the
218 additional attraction to the ends of the I-BAR domain for only a small percentage of the

219 membrane beads. Consistent with our observation on GUVs, we found that I-BAR domain
220 clustering occurs on both PS-like (i.e., 0% of PIP₂-like membrane) and PIP₂-like membranes (2%
221 and 5% of PIP₂-like membranes, Fig. 2D, Top). Moreover, we found that the addition of PIP₂-
222 like membrane beads increases the aggregation of I-BAR domains. Without PIP₂-like membrane
223 beads, we found a high probability of free or small aggregates containing less than five I-BAR
224 domains. In the presence of PIP₂-like membrane beads, there is a significant decrease in free or
225 small aggregates and a corresponding increase of larger aggregates with five or more I-BAR
226 domains (Fig. 2D Top, Movie S2, and Fig. 2E). Importantly, it has been shown that I-BAR
227 domains can induce stable PIP₂ microdomains upon membrane binding (31, 49). Indeed, we
228 found an enrichment of PIP₂-like membrane beads near I-BAR domain aggregates (Fig. 2D,
229 Bottom, blue dots, and Movie S3). In the 2% PIP₂-like membranes, we found 27% of
230 neighboring membrane beads are PIP₂-like, which is an approximate 14 times enrichment of
231 PIP₂-like membrane beads around the I-BAR domain aggregates compared to the total 2%
232 concentration of PIP₂-like beads on the membrane. Similarly, in the 5% PIP₂-like membranes,
233 we found 33% of neighboring membrane beads are PIP₂-like, which is a 6.7 times enrichment of
234 PIP₂-like membrane beads to the aggregates compared to the total 5% concentration of PIP₂-like
235 membrane beads. The enrichment of PIP₂-like membrane beads around I-BAR domain
236 aggregates depletes PIP₂ in the bulk membrane that is not adjacent to an I-BAR domain
237 aggregate. In other words, the PIP₂ percentages in the membrane adjacent to the I-BAR domain
238 aggregates are enriched to 27% and 33% while the rest of the membrane is depleted to 0.7% and
239 3.4% of PIP₂-like membrane beads for 2% and 5% PIP₂-like membrane systems, respectively.
240 Our results thus indicate positive feedback of the assembly of I-BAR domain aggregates
241 mediated by PIP₂ and the enrichment of PIP₂ around the aggregates facilitate further I-BAR
242 domain recruitment. Altogether, our simulation and experimental reconstitution results indicate
243 the key role of PIP₂ in IRSp53 clustering on membranes.

244 It was reported that in bulk, as well as on small vesicles containing PIP₂, IRSp53 and
245 VASP interact directly via their SH3 domain and PR domain, respectively (16). We thus
246 examined this interaction in our reconstituted system. We purified full-length human VASP and
247 labelled it with AX488 dyes (AX488-VASP). When incubating PIP₂-GUVs with AX488-VASP
248 (4 nM of VASP tetramer) together with unlabeled full-length IRSp53 (16 nM of IRSp53 dimer),
249 we observed that VASP is recruited on GUV membranes (Fig. 2F Top, arrows), which is not the
250 case in the absence of IRSp53 (Fig. 2F, Bottom). Consistently, we observed VASP recruitment
251 on GUV membranes when replacing full-length IRSp53 with a truncated version of IRSp53 that
252 contains the I-BAR domain and the SH3 domain of IRSp53 (Fig. S4 A); in contrast, no VASP
253 recruitment was observed when replacing full-length IRSp53 with the isolated I-BAR domain of
254 IRSp53 (Fig. S4 B). These findings are consistent with the previous observations that IRSp53
255 and VASP interact directly in solution, on model membranes, and in filopodia (16, 17, 19).
256 Notably, we found clusters of VASP on GUV membranes (Fig. 2F, arrows) that are reminiscent
257 of what has been observed in live cells (Fig. 1B and (16)). Furthermore, for GUVs having
258 IRSp53-generated membrane tubules, we observed that VASP is recruited in these tubules (Fig.
259 S5). Our results indicate that IRSp53 can recruit VASP into clusters on the relatively flat GUV
260 surfaces and to negatively curved membrane tubes, a characteristic of filopodial membranes.

261 **Self-assembly of IRSp53, VASP, fascin and actin on PIP₂-membranes generates actin-filled**
262 **membrane protrusions**

263 We next assessed if IRSp53, VASP, actin and fascin can self-organize on PIP₂-
264 membranes to drive protrusion formation, as hypothesized above. We kept the bulk
265 concentration of IRSp53 and VASP relatively low (16 nM of IRSp53 dimer and 4 nM of VASP
266 tetramer) to allow the formation of IRSp53-VASP clusters on PIP₂-membrane as seen in cells.
267 To visualize actin, we used AX488-labelled globular actin (G-actin, ~10% - 27% AX488
268 labelled, total actin concentration 0.5 μM). To ensure that actin polymerization occurs at the
269 membrane only, as in cells, and not in solution, we included capping protein (CP, 25 nM) and
270 profilin (0.6 μM) in the protein mixture. Capping protein binds to the barbed ends of filamentous
271 actin (F-actin) with high affinity ($K_d = 0.1$ nM) and inhibits F-actin elongation in the bulk (1,
272 50). In cells, the majority of G-actin is associated with profilin ($K_d = 0.1$ μM) (51, 52), which
273 suppresses spontaneous actin nucleation in the bulk. Additionally, it was shown that at high ionic
274 strengths, profilin is required for VASP to be more effective in actin polymerization (36).
275 Finally, to introduce actin filament bundling as in filopodia, we used fascin at 250 nM (11).

276 By performing pyrene actin polymerization assays, we verified that in our experimental
277 conditions VASP increases actin polymerization in the presence of profilin, and CP suppresses
278 F-actin elongation (Fig. S6, solid lines). To verify the presence of actin filaments in our
279 reconstitution systems, we performed some experiments using unlabeled G-actin and included
280 AX488 phalloidin (0.66 μM) in the protein mixture, given that AX488 phalloidin binds to F-
281 actin and its fluorescence is higher on F-actin than in the bulk (53). We verified by pyrene actin
282 assays that the actin polymerization activity of VASP is preserved when phalloidin is present (by
283 comparing the green dashed curve and the black dashed curve in Fig. S6). Also, the suppression
284 of actin polymerization by CP is preserved in the presence of phalloidin, as indicated by the
285 initial actin polymerization rates with 56 pM polymerized actin/sec and 134 pM polymerized
286 actin/sec in the presence and absence of CP, respectively (by comparing the initial slopes of the
287 dashed pink and dashed green curves in Fig. S6. See the Materials and Methods for details of the
288 calculation). Finally, the actin bundling activity of fascin was verified by performing two
289 independent assays: an actin-fascin co-sedimentation assay at low centrifugation speed, showing
290 most of actin filaments were present in the pellet fraction only when fascin was present (Fig. S7
291 A), and an actin-fascin co-polymerization assay observed by confocal microscopy, showing
292 bundles of actin filaments only when fascin was present (Fig. S7 B).

293 By incubating PIP₂-GUVs with IRSp53, VASP, actin, fascin, CP and profilin, we
294 observed inward membrane tubes filled with actin on the GUVs (Fig. 3A). The tubes are not
295 static, but move rapidly inside GUVs (Movie S4 and S5). In addition to the tubes, there are actin
296 signals on GUV membranes, indicating the formation of an actin shell on the membrane (Fig.
297 3A). On average, 32% of the GUVs had tubes, and 93% of these tube-positive GUVs had at least
298 one tube filled with actin (N = 140 GUVs, n = 3 sample preparations, Fig. S8 A). By using
299 AX488 phalloidin together with unlabeled G-actin, we observed clear AX488 phalloidin signals
300 inside tubes as well as on GUV membranes, confirming the presence of F-actin (Fig. 3B, Movie
301 S6 and S7). In the presence of phalloidin, on average, 55% of the GUVs had at least one actin-
302 filled tube (N = 366 GUVs, n = 7 sample preparations, and Fig. S8, B and C). Finally, we
303 verified the presence of IRSp53, VASP and fascin in actin-filled membrane protrusions by using
304 AX488 labelled IRSp53, VASP and fascin. Regardless of phalloidin, we observed that VASP
305 and fascin are present throughout the protrusions while IRSp53 is either present throughout the
306 protrusions or enriched at the tips of protrusions where the fluorescence signal of actin is
307 somewhat weaker compared to the rest of the protrusion (Fig. S9).

308 Using the fluorescence signals of either AX488 actin monomers or AX488 phalloidin, we
309 quantified that on average there are ~2 and ~11 actin filaments in the tubes, respectively (Fig.
310 S10 A). Although the estimated numbers of filaments in tubes are lower than those in filopodia
311 (typically 10 – 30 filaments) (6, 7), they are comparable to theoretical predictions in which 2 – 3
312 growing filaments are required to overcome membrane restoring force to generate extensions
313 (54). Moreover, the membrane enclosing the actin bundles was predicted to stabilize the bundle
314 against buckling in which the minimum number of filaments required for protrusion stability is
315 four (55). In our reconstitution system, given the previously reported ability of the I-BAR
316 domain in stabilizing membrane tubes (32), we anticipate that the presence of IRSp53 in the
317 actin-filled tubes could contribute to their stability. We noted that the higher number of filaments
318 in the presence of phalloidin could be due to the previously reported effect of phalloidin in
319 promoting actin nucleation (56). Besides, phalloidin was shown to increase the persistence length
320 of actin filaments, thus effectively making them stiffer (57). We thus assessed the orientational
321 properties of the actin-filled tubes by performing image analysis (see Materials and Methods for
322 details). As shown in Fig. S10, color-coded orientation maps showed the presence of dominant
323 orientation directions of the tubes in the presence of phalloidin but not in its absence.
324 Consistently, in the presence of phalloidin, higher coherency values, which indicate one
325 dominant orientation of local structures, can be seen in places where the protrusions are, as
326 readily visible by the white patches in the coherency map (indicated by arrows in Fig. S10, D
327 and E). Thus, consistent with the higher number of actin filaments in the tubes and the above-
328 mentioned side effects of phalloidin, our results show that the actin-filled tubes are straighter in
329 the presence of phalloidin

330 Besides tubes, we observed membrane deformations on the GUV membranes
331 independent of the presence of phalloidin (Fig. S11, arrows). The deformed bulge shape of the
332 GUV membranes is reminiscent of membrane deformation driven by the I-BAR domain of
333 IRSp53 on GUVs (58), and indicates the presence of pushing forces acting on the GUV
334 membranes towards the interior of the GUVs. Altogether, our results show that IRSp53, VASP,
335 actin and fascin can spontaneously organize locally on PIP₂-membranes to generate actin-based
336 membrane protrusions.

337 **IRSp53 is indispensable for protrusion formation by recruiting VASP to facilitate actin** 338 **polymerization in protrusions**

339 To reveal the contribution of individual protein components in the generation of actin-
340 filled membrane protrusions, we performed loss-of-function assays by removing one protein
341 component at a time. To ensure that protein activities are identical in the loss-of-function assays,
342 we performed paired experiments in which we used the same batches of GUVs and protein
343 stocks in each independent sample preparation. Then, in each sample preparation, we compared
344 the efficiencies of the generation of actin-filled tubes in the absence of a protein of interest, and
345 the reference condition where all the proteins are present. To this end, we counted the number of
346 GUVs with and without tubes, and if an actin signal was readily detected in at least one or more
347 tubes of the GUVs.

348 When IRSp53 was absent, we found none of the GUVs had tubes (Fig. 3C, N = 57
349 GUVs, n = 3 sample preparations, in the presence of AX488 phalloidin). Thus, in our
350 experimental condition, IRSp53 is essential for the generation of actin-filled membrane tubes. In
351 the absence of VASP, we observed a nearly complete lack of actin signal on the GUV
352 membranes (Fig. 3D and Fig. S12 A), and nearly no GUV had actin-filled tubes (out of a total of

353 116 GUVs, $n = 3$ sample preparations, only 8 GUVs were found with tubes, in which only 1
354 GUV had actin-filled tubes; Fig. 3E and Fig. S12 B). Our observations thus indicate the key role
355 of VASP in the generation of membrane tubes, via recruiting actin on GUVs. Given that VASP
356 is an actin polymerase, we assessed if VASP facilitates actin polymerization in tubes by using
357 AX488 phalloidin. Indeed, we observed that due to the presence of VASP, there is an increase in
358 the number of GUVs having actin-filled tubes (Fig. S13, A and B) and an increase in the number
359 of actin filaments in the tubes (Fig. S13 C). We note that in the presence of phalloidin, there are
360 actin-filled tubes on GUVs even when VASP is absent (Fig. S13 D). This observation indicates
361 that phalloidin aids actin polymerization even when CP and profilin are present. To understand
362 how phalloidin influences actin polymerization in our system, we performed pyrene actin
363 polymerization assays. We observed that phalloidin enhances VASP-mediated actin nucleation
364 (Fig. S6, compare the magenta solid and dashed curves), consistent with a previous report (56). It
365 was shown in vitro that in the bulk, IRSp53 (after being activated by Eps8) and its I-BAR
366 domain can interact with F-actin and induce actin bundle formation (20, 23). Thus, in the
367 absence of VASP, phalloidin facilitates actin polymerization, and IRSp53 recruits F-actin to
368 GUV membranes, resulting in the formation of actin-filled tubes. Consistently, we found that the
369 percentages of GUVs having actin-filled tubes is higher in the presence of phalloidin compared
370 to its absence (Fig. S8 C). Taken together, our results show that through IRSp53-driven
371 recruitment to PIP₂-membranes, VASP plays a key role in protrusion generation via its actin
372 polymerization activity.

373 Biophysical studies using reconstitution systems and theoretical modelling have revealed
374 how the interplay between the mechanical properties of membranes and actin bundles/networks
375 determines the formation of actin-driven membrane protrusions (12, 59, 60). Given that actin's
376 role in the initiation of IRSp53-driven cellular protrusions is not fully understood (18), we
377 assessed if actin facilitates IRSp53-based protrusion formation using our reconstitution systems.
378 In the absence of actin, we observed 2% to 34% of GUVs having tubes (Fig. 3 F and G,
379 regardless of phalloidin). These tubes were generated by IRSp53 since when IRSp53 is absent,
380 no GUV has tubes (Fig. 3C). Notably, we observed a global increase in the amount of GUVs
381 having tubes due to the presence of actin (Fig. 3 F and G). This effect is more pronounced when
382 phalloidin is present (Fig. 3G, $N = 127$ and 208 total GUVs, without and with actin, respectively.
383 $n = 4$ sample preparations). In the absence of phalloidin, in a sample preparation where there was
384 a relatively high amount of GUVs with tubes in the absence of actin (34.1%, $N = 41$ GUVs), the
385 addition of actin did not aid tube generation (25.6%, $N = 39$ GUVs). However, in experimental
386 sets where the amount of GUVs with tubes was low (less than 10%) in the absence of actin, the
387 addition of actin increased the amount of GUVs with tubes (Fig. 3F, $N = 99$ and 101 total GUVs,
388 without and with actin, respectively, $n = 2$ sample preparation). Together, these results indicate
389 that actin contributes to IRSp53-based tube generation. Furthermore, given that phalloidin
390 facilitates actin nucleation on membranes in our system, our results point out the essential role of
391 actin nucleation to enhance actin's function in protrusion formation in cells.

392 **Fascin is not required for protrusion generation but enhances protrusion elongation and** 393 **stability**

394 Given that fascin is the specific actin bundler in filopodia, we assessed its role in
395 protrusion formation. In the presence and absence of fascin, we did not observe significant
396 differences considering the number of GUVs having actin-filled tubes, regardless of the absence
397 (Fig. 4A and Fig. S14 A) or presence of phalloidin (Fig. S14, B and C). Furthermore, there is no

398 significant difference in the amount of F-actin in tubes in the presence and absence of fascin
399 (Fig. S14 D). Due to the rapid movement of tubes inside GUVs, we could not characterize the
400 dynamics of tube generation and elongation. We thus assessed how fascin affects protrusion
401 dynamics in live cells. To this end, we performed experiments using Rat2 cells that expressed
402 eGFP-tagged IRSp53 and mCherry-tagged fascin (Fig. 4B and Movie S8). We observed that the
403 recruitment of fascin in IRSp53-based protrusions coincides with their elongation (Fig. 4 C and
404 D). By tracking IRSp53-based protrusions, we also found that fascin significantly increases the
405 growth rates of the protrusions (Fig. 4D), and that its over-expression decreases the frequency of
406 their retraction (Fig. 4E). Thus, our in vitro reconstitution data demonstrate that actin
407 polymerization facilitates the formation of IRSp53-dependent membrane protrusions, while the
408 cell data suggest that fascin increases the growth rate and enhances the stability of these
409 protrusions.

410 **IRSp53 regulation in cells revealed by membrane nanotube pulling experiments**

411 Our results obtained using the reconstituted system demonstrate the function of IRSp53
412 in the generation of actin-filled membrane protrusions and show that IRSp53 is primed to self-
413 assemble into clusters that drive the association of downstream partners (e.g., VASP, fascin) for
414 protrusion growth. However, it remains unclear how cells regulate the activity of IRSp53 to
415 control for instance protrusion formation at specific membrane locations. Indeed, with the
416 abilities of IRSp53 to sense membrane curvature and promote actin assembly mediated by
417 VASP, we expect that any local deformation of the plasma membrane could induce filopodial
418 growth. To reveal IRSp53's possible regulation directly in cellulo, we generated artificial
419 protrusions having identical topologies to filopodia by pulling membrane nanotubes (i.e., tethers)
420 from the plasma membrane of Rat2 fibroblasts using optically trapped polystyrene beads and
421 micromanipulation (Fig. 5A). The pulled nanotubes serve as tractable models to directly assess
422 the recruitment of IRSp53 into tubular geometries and the possible actin assembly inside these
423 nanotubes as a consequence of IRSp53 enrichment. Cells were transfected with either IRSp53's
424 I-BAR domain (I-BAR-eGFP), its putative membrane deforming and curvature sensitive region,
425 or the full-length IRSp53 protein (IRSp53-eGFP). The plasma membrane was exogenously
426 labelled using the lipophilic Cell MaskTM Deep Red stain. Rat2 I-BAR-eGFP expressing cells
427 show strong I-BAR-eGFP fluorescence in pulled nanotubes (Fig. 5B) that was visible after
428 nanotube formation (Fig. S15 and Movie S9), suggesting an innate preference of the I-BAR
429 domain to sort into these nanotubes. Protein enrichment in the nanotube was quantified by
430 determining the sorting parameter S , which corresponds to the ratio of the protein intensity in the
431 nanotube (at a given pixel) relative to the protein's average intensity measured in the plasma
432 membrane (i.e., the bulk). This nanotube/membrane ratio for the protein is further normalized by
433 the same ratio for a lipid marker (here the Cell MaskTM Deep Red stain) to correct for cell-to-cell
434 signal differences in the membrane and protein fluorescence channels (e.g., differences in
435 expression levels, staining efficiencies). This calculation ultimately results in a color-coded
436 image of S values (we term a S map) from which we then can determine a mean enrichment
437 value for a given nanotube (S_{avg}) (Fig. 5C; see Materials and Methods for further details). Note
438 that S_{avg} values greater than 1 indicate preferential protein enrichment for a given nanotube. We
439 measured the S_{avg} of the I-BAR domain for multiple nanotubes ($N = 19$ nanotubes) and
440 determined an ensemble average of 4.5 (Fig. 5D). This value is similar to previously reported
441 sorting values obtained from nanotubes pulled from GUVs encapsulating the purified I-BAR
442 domain (32).

443 Contrary to the robust enrichment of the I-BAR domain, we observed a more complex
444 behavior for the sorting of the full-length IRSp53 protein in nanotubes that is dependent upon the
445 local cellular membrane activity near the sites of the nanotubes. Pulled nanotubes from IRSp53-
446 eGFP expressing cells show strong IRSp53 signal only when the nanotube was pulled near zones
447 exhibiting “active” processes of membrane remodeling (Fig. 6A), such as lamellipodia and
448 membrane ruffles, which were frequently found at the cell leading edge. Similar to the fast
449 arrival of the I-BAR domain in pulled nanotubes (Fig. S15), we observed a rapid stabilization of
450 IRSp53 intensity in pulled nanotubes within 30 sec (Fig. S16). However, no IRSp53-eGFP
451 fluorescence was observed in nanotubes when pulled from “non-active” zones (Fig. 6B), such as
452 those found near the cell trailing edge. Subsequent imaging over time showed that nanotubes
453 pulled from “active” and “non-active” regions stably retained either the presence or absence of
454 IRSp53, respectively, indicating an equilibrium situation was reached in our measurements (Fig.
455 S17). As seen in Fig. 6C, nanotube S_{avg} values for IRSp53 near “active zones” (N = 13
456 nanotubes) averaged 2.8 and was comparable to the I-BAR domain case (Fig. 5D). However, S_{avg}
457 values for IRSp53 near “non-active zones” (N = 20 nanotubes) averaged 0.2, indicating an
458 exclusion of the protein from the nanotubes. The dichotomy of sorting behaviors for IRSp53 is
459 completely opposite to the results we observed for the I-BAR domain, where the I-BAR domain
460 sorting is consistently stronger and did not depend on the cellular region where the nanotube
461 pulling was performed. Together, our results highlight that IRSp53’s curvature sensing ability is
462 innately part of the I-BAR domain (32, 33) and further suggest that cellular regulation
463 mechanisms are at play in modulating IRSp53’s activity.

464 We observed that the sorting of IRSp53 has a direct consequence on the assembly of F-
465 actin within the nanotubes. Nanotubes with high IRSp53 sorting (Fig. 6D) exhibited
466 characteristic signatures of F-actin in the measured force profile and behaviors mimicking those
467 of true filopodia. The observed bead displacement within the optical trap (i.e., a rise in the force,
468 ΔF) indicates that F-actin development has reached the end of the nanotube, allowing retrograde
469 and contractile forces to transmit from the cell body to the bead. The magnitude of the force
470 peaks generates traction forces of 5–10 pN (Fig. 6E) and are of the same order of magnitude as
471 those reported for true filopodia (61, 62). Additionally, events of nanotube curling (i.e., helical
472 buckling) were also frequently observed (Fig. S18), as previously shown for filopodia (62). In
473 essence, IRSp53-positive nanotubes eventually mature into a “pseudo” filopodium. However,
474 nanotubes with no IRSp53 enrichment (Fig. 6F) exhibit a constant force profile and consequently
475 no F-actin development was observed. Consistent with our GUV results, in IRSp53-positive
476 nanotubes, we also observed the recruitment of both VASP and fascin (Fig. S19 and Movie S10
477 and S11) in cells co-expressing the proteins of interest, confirming bundled F-actin is indeed
478 present within the core of the nanotube. Together, our tube pulling results show that though
479 IRSp53 is curvature sensitive through its I-BAR domain, its activity is tightly controlled within
480 cells to constrain local F-actin development for protrusion generation.

481

482 Discussion

483 How cells control the formation of protrusions such as filopodia and microvilli at specific
484 membrane locations remains largely unclear. Using in vitro reconstitution systems, we
485 demonstrated for the first time that a minimum set of proteins comprised of the membrane
486 curvature sensor IRSp53, the actin polymerase VASP, and actin can spontaneously organize to

487 generate actin-based membrane protrusions. By performing loss-of-function assays, we
488 investigated the function of proteins in protrusion formation. We demonstrated that IRSp53 is the
489 essential player in protrusion generation by recruiting VASP to promote local actin assembly.
490 Thus, by using a precisely controlled reconstitution system, our study provides a strong support
491 to the previously proposed mechanism of IRSp53-VASP-driven filopodia generation that was
492 largely based on cell biology studies (16, 17).

493 A key finding of our in vitro assay is that purified full-length IRSp53 is active in the
494 absence of its activators such as Cdc42. Our results indicate that once IRSp53 binds to the
495 plasma membrane, it can self-assemble into clusters and readily recruit VASP to generate actin-
496 filled protrusions. Indeed, it was reported that when expressing a non-regulated constitutively
497 active mutant of IRSp53 in cells, an aberrant explosive formation of filopodia was observed (21).
498 Thus, to prevent generating unwanted protrusions, cells must have a tight regulation of IRSp53,
499 i.e., how much, when, and where to activate IRSp53. To reveal the regulation of IRSp53 activity
500 in cells, we generated filopodia-like membrane geometries in live cells by pulling membrane
501 nanotubes from the plasma membrane. We discovered that IRSp53 can only be recruited to
502 nanotubes pulled from active membrane regions of the cell (e.g., where the membrane exhibits
503 nearby dynamic ruffling, lamellipodia extension, and filopodia formation) but not from regions
504 where no membrane remodeling was observed and thus IRSp53 is presumed inactivated and
505 unable to bind to the plasma membrane to exert its function. Moreover, actin polymerization
506 extending throughout the IRSp53-enriched nanotube was detected, creating in essence a
507 “pseudo” filopodium that was positive for VASP and fascin. Our finding is consistent with the
508 location of IRSp53’s activator Cdc42 at the front edge of migratory cells where ruffling and
509 protrusions develop (63, 64), and with IRSp53’s function in inducing Rac-dependent membrane
510 ruffling (26, 29). Importantly, our results indicate that to finely regulate IRSp53 activity, it is
511 probably even more important to keep IRSp53 inhibited such that its binding to membranes is
512 prevented. Indeed, it was shown that the binding of 14-3-3 to IRSp53 counteracts the activation
513 by Cdc42 and other downstream cytoskeleton effectors (21). Notably, 14-3-3 binding to
514 phosphorylated IRSp53 keeps it in an inhibited state, resulting in impaired filopodia formation
515 and dynamics (21, 29, 30). Collectively, our results reflect a finely tuned regulatory mechanism
516 where IRSp53 is kept in its inhibition state by phosphorylation and binding to 14-3-3, and
517 activated by binding to activators such as Cdc42. Controlling IRSp53 by three different signaling
518 pathways, phosphorylation, Cdc42 binding and PIP₂ binding thus ensures its precise spatial-
519 temporal regulation at the plasma membrane such that IRSp53 is activated only at specific
520 regions of cells.

521 It was shown previously that PIP₂-binding by IRSp53’s I-BAR domain is required for the
522 generation of plasma membrane protrusions (25). Indeed, our in vitro work and coarse-grained
523 simulation results showed that PIP₂ is key for the assembly of these IRSp53 clusters: higher PIP₂
524 concentration results in larger I-BAR domain clusters. Importantly, our simulation results
525 showed an at least 7-fold enrichment of PIP₂ around the I-BAR domain clusters. Our finding is
526 consistent with the previous reports of BAR and I-BAR domains inducing local and stable PIP₂
527 clusters (31, 49, 65). Notably, it was proposed that these PIP₂ clusters are transiently interacting
528 with BAR domains such that PIP₂ in the clusters is available for the recruitment of downstream
529 partners having PIP₂ binding motifs (31, 49, 65). We thus propose that besides specific protein-
530 protein interactions, IRSp53-induced PIP₂ clusters could facilitate not only the further
531 recruitment of IRSp53 but also other proteins such as actin nucleation promoting factors, actin

532 polymerases and actin-binding proteins for protrusion formation and regulation (17, 24, 27, 66-
533 68).

534 We observed that clusters of IRSp53 preceding those of VASP on the plasma membrane
535 at the onset of filopodia formation. Our observations are in agreement with what reported by
536 other studies using MEF cells and COS-7 cells (16, 21). VASP clustering has been shown to be
537 an important factor to increase the processivity of VASP for actin filament elongation (37, 39).
538 Consistently, using reconstitution systems, we showed that VASP clustering via IRSp53
539 efficiently polymerizes actin on PIP₂-membranes and in protrusions. Moreover, we observed an
540 increased number of actin filaments in protrusions when VASP is present. Notably, VASP
541 clustering has also been attributed to the formation of another type of membrane protrusion
542 called microspikes. As shown previously, despite containing actin bundles, microspikes differ
543 from filopodia by being shorter protrusions, which are generated from and are largely embedded
544 in the lamellipodial actin filament network. By focusing on the formation of microspikes, recent
545 studies using B16F1 cells showed that VASP clustering is initiated by lamellipodin and myosin-
546 X, but not by IRSp53 (42). These studies and our work thus suggest that VASP requires specific
547 membrane-interacting partners to bring it to membranes and induce its clustering to drive
548 outward membrane deformation. The differences in the mechanisms of recruiting VASP on
549 membranes may be also cell-type dependent, i.e., cells may utilize different pathways to induce
550 membrane deformation via VASP. Of note, in our current study, IRSp53 and VASP were
551 overexpressed in cells, and the elevated expression levels could potentially influence the
552 regulation of different molecular machineries involved in the generation of membrane
553 protrusions. In the future, it will be important to study the roles of different proteins in VASP
554 clustering during both filopodia and microspike formation in different cell-types by expressing
555 proteins of interest at levels comparable to their endogenous counterparts. This would allow to
556 decipher how and under which physiological conditions cells use different VASP clustering
557 pathways to generate distinct membrane protrusions.

558 In our in vitro GUV assays, we observed that IRSp53 is either present throughout the
559 actin-filled protrusions or enriched at the tips of the protrusions. This observation is in agreement
560 with the localization of full-length IRSp53 in cellular filopodia using super-resolution
561 microscopy (69). Consistently, given that IRSp53 is responsible for the recruitment of VASP to
562 GUV membranes, we observed that VASP is present throughout the protrusions. Along the same
563 line, in our cell-tether pulling experiments, we observed that VASP is distributed throughout the
564 tethers. These observations are different from the reported localization of VASP in filopodia,
565 where VASP is mostly found at the tips of filopodia and to a lesser extent also along the shaft of
566 filopodia. Of note, in contrast to the very simplified GUV systems, the formation of filopodia in
567 cells involves an overwhelmingly large set of other proteins (70). We thus speculate that in cells
568 after IRSp53 initiates VASP-dependent actin elongation, other binding partners of VASP that are
569 members of the filopodial tip complex could restrict the localization of VASP to be at the
570 filopodial tip. Potential VASP partners could be Myosin-X and formin mDia2, which were
571 shown to cooperate with VASP in filopodia formation (43, 71, 72). Our in vitro work provides
572 direct evidence that actin filament assembly facilitates IRSp53-based protrusion generation. This
573 is consistent with what was proposed previously based on EM and cell biology experiments (18).
574 In our assay, actin assembly is driven by VASP, given that when VASP was eliminated, we
575 observed nearly no actin signal on GUVs (Fig. 3D). We anticipate that other actin assembly
576 factors can play similar roles as VASP. Indeed, it was reported that IRSp53 interacts directly
577 with formins, such as mDia1, in filopodia formation (27). Moreover, actin nucleation promoting

578 factors WAVE2 and N-WASP were shown to synergize with IRSp53 to generate filopodia (17,
579 27). In our in vitro system, fascin is not essential for protrusion initiation. However, by observing
580 the dynamics of filopodia in Rat2 cells, we found that fascin enhances the elongation rate and
581 stability of filopodia, consistent with the previous observation in cells that used RNA
582 interference of fascin (4-7). Our result thus supports the notion that fascin mechanically
583 strengthens filopodia by bundling actin filaments together, thus facilitating filopodial extension
584 and stabilization (4-7). Notably, Eps8, another actin bundler, has been reported to form a
585 complex with IRSp53 during filopodia formation (20). Besides bundling, Eps8 can cap the
586 barbed end of actin filaments. The dual function of Eps8 on actin filaments is fine-tuned in cells,
587 allowing cells to generate filopodia via different signaling networks depending on cellular
588 contexts (20). To understand how the abovementioned proteins regulate dynamic filopodia
589 formation, elongation, retraction, and force generation, future work is required to elucidate the
590 interplay between these proteins in the context of protrusion formation.

591 Altogether, our work demonstrates that IRSp53 is an efficient protrusion initiator: once
592 being activated, IRSp53 readily triggers the cascade of protrusion formation. Thus, to avoid the
593 uncontrolled formation of protrusions, it is critical for cells to have a strict and precise control on
594 when and where to activate or unlock IRSp53 from its inhibitory state

595

596 **Materials and Methods**

597 Pyrene actin polymerization assay

598 Polymerization assays were based on measuring the fluorescence change of pyrenyl-labeled G-
599 actin ($\lambda_{exc}=365$ nm, $\lambda_{em}=407$ nm). Experiments were carried out on a Safas Xenius
600 spectrofluorimeter (Safas, Monaco). Polymerization assays were performed in buffer containing
601 60 mM NaCl, 1 mM MgCl₂, 0.2 mM EGTA, 0.2 mM ATP, 10 mM DTT, 1 mM DABCO, 5 mM
602 Tris pH 7.5 and 0.01% NaN₃ in the presence of actin (2 μ M, 5% pyrenyl labeled), profilin (2.4
603 μ M), VASP (15 nM in tetramer), CP (25 nM) and phalloidin (2 μ M).

604 To reveal the upper limit of the fluorescence intensity for the pyrene actin polymerization, we
605 performed positive control experiments of (1) actin + spectrin-actin seeds, (2) actin alone, (3)
606 actin + phalloidin. As shown in Fig. S6, the sample of actin + spectrin seeds reaches completion
607 with the fluorescence intensity of a value of 50 A.U. after around 1000 sec.

608 To verify that in the presence of VASP, phalloidin does not inhibit/prevent CP from suppressing
609 F-actin elongation, we estimated the initial actin polymerization slopes of two conditions: (1)
610 profilin actin + VASP + CP + phalloidin (dashed pink curve in Fig. S6 B) and (2) profilin actin +
611 VASP + phalloidin (dashed green curve in Fig. S6 B). We considered that the fluorescence
612 reached the plateau value of 48.2 A.U. (obtained by using spectrin-actin seeds as shown in Fig.
613 S6 A) correspond to 1.9 μ M of actin, i.e., the total concentration of actin subtracts 0.1 μ M (the
614 critical concentration for actin polymerization). We obtained the following results: (1) profilin
615 actin + VASP + CP + phalloidin (dashed pink curve): 56 pM polymerized actin / sec, and (2)
616 profilin actin + VASP + phalloidin (dashed green curve): 134 pM polymerized actin / sec. We
617 note that

618 GUV experiments

619 **Reagents**

620 Brain total lipid extract (TBX, 131101P), brain L- α -phosphatidylinositol-4,5-bisphosphate (PIP₂,
621 840046P), 18:1 PS (DOPS, 840035), and Cy5-PC (850483) were purchased from Avanti Polar
622 Lipids. BODIPY-TR-C5-ceramide, (TR-ceramide, D7540) was purchased from Invitrogen.
623 Alexa Fluor 488- and Alexa Fluor 594-tagged phalloidin (AX488 phalloidin and AX594
624 phalloidin) were purchased from Interchim. β -casein from bovine milk (>98% pure, C6905) and
625 other reagents were purchased from Sigma-Aldrich.

626 **GUV preparation**

627 The lipid mixture used contained total brain extract supplemented with 5 mol% PI(4,5)P₂ at 0.5
628 mg/mL dissolved in chloroform. To visualize GUV membranes, the lipid mixture was
629 supplemented with either 0.5 mol% TR-ceramide or 0.5 mol% Cy5-PC.

630 GUVs were prepared by using the polyvinyl alcohol (PVA) gel-assisted vesicle formation
631 method as previously described (73). Briefly, a PVA gel solution (5 %, w/w, dissolved in 280
632 mM sucrose and 20 mM Tris, pH 7.5) warmed up to 50 °C was spread on clean coverslips
633 (20mm × 20mm). The coverslips were cleaned by ethanol and then ddH₂O twice. The PVA-
634 coated coverslips were incubated at 50°C for 30 min. Around 5 μ L of the lipid mixture was
635 spread on the PVA-coated coverslips and then dried under vacuum at room temperature for 30
636 min to remove the chloroform solvent. The coverslips were then placed in a petri dish and
637 around 500 μ L of the inner buffer was pipetted on the top of the coverslips. The inner buffer
638 contains 50 mM NaCl, 20 mM sucrose, 20 mM Tris pH 7.5. The coverslips were kept at room
639 temperature for at least 45 min, allowing GUVs to grow. Once done, we gently “tapped” the
640 bottom of the petri dish to detach GUVs from the PVA gel. The GUVs were collected using a 1
641 mL pipette tip with its tip cut to prevent breaking the GUVs.

642 **Protein purification and labelling**

643 Muscle actin (Uniprot# P68135) was purified from rabbit muscle and isolated in monomeric
644 form in G-buffer (5 mM Tris-Cl, pH 7.8, 0.1 mM CaCl₂, 0.2 mM ATP, 1 mM DTT, 0.01%
645 NaN₃) as previously described (74). Actin was labeled with Alexa 488 or Alexa 594 succinidyl
646 ester-NHS (75).

647 The genes encoding full-length human IRSp53 (Uniprot #Q9UQB8, Homo sapiens) and the I-
648 BAR-SH3 domain of IRSp53 (Uniprot #Q9UQB8, Homo sapiens, amino acid 1-452), and the
649 full length human VASP (Uniprot #P70460, Homo sapiens) were provided by Prof. Roberto
650 Dominguez (University of Pennsylvania), and Prof. Jan Faix (Hannover Medical School),
651 respectively, and sub-cloned into the pGEX-6P-1 vector (Cytiva). The expression plasmids were
652 transformed into BL21 (DE3) competent cells. A single colony was resuspended in 10 mL LB
653 medium containing 100 μ g/mL ampicillin, and cultured at 37°C overnight. Then, the starter
654 culture was inoculated into 1 L LB medium containing 100 μ g/mL ampicillin, and cultured at
655 37°C until OD₆₀₀ reached 0.6. The protein expression was induced with 0.2 mM isopropyl-D-1-
656 thiogalactopyranoside (IPTG) overnight at 15°C. After harvesting the cells, the pellets were
657 resuspended and sonicated in a buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 1
658 mM EDTA, 1 mg/mL lysozyme, 1% Triton X-100, 1 mM PMSF, and 1 mM DTT, followed by
659 affinity purification with GSTrap FF (Cytiva). The GST-tag was removed with PreScission
660 protease (Cytiva). The proteins were concentrated to 50–100 μ M using Amicon Ultra centrifugal
661 filters (Merck). For protein labelling, Alexa Fluor maleimides (Thermo Fisher Scientific) were
662 added to the protein solution, and the reaction was allowed to proceed at 4°C overnight in the
663 dark to protect it from light. The labelled proteins were further purified and separated from free

664 Alexa Fluor dye using a Superdex 75 10/300 GL gel filtration column (Cytiva) using either an
665 ÄKTA protein purification system (Cytiva) or NGC Chromatography System (Bio-Rad). The
666 proteins were concentrated using Amicon Ultra centrifugal filters (Merck) by replacing the
667 buffer with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM DTT, then 0.1 % (w/v)
668 methylcellulose (M0512, Sigma, 4000 cPs) was added. The purified proteins were frozen in
669 liquid nitrogen and stored at -80°C before use. The labelling efficiency of Alexa 488 IRSp53
670 (dimer) was 0.94, and that of Alexa 488 VASP (tetramers) was 3.0. The purification of the
671 isolated mouse I-BAR domain of IRSp53 (Uniprot #Q8BKX1, mouse, amino acid 1-250) was
672 performed as previously described (31).

673 Recombinant human profilin I was expressed in BL21 (DE3) Star competent bacterial cells and
674 purified as described (76). The plasmid for the expression of mouse capping protein $\alpha 1\beta 2$
675 (Uniprot #P47753 ($\alpha 1$) and #P47757 ($\beta 2$), mouse) was cloned in a pRSFDuet-1 vector,
676 providing a 6xHis tag at the N terminus of the $\alpha 1$ subunit. Mouse capping protein $\alpha 1\beta 2$ was
677 expressed in BL21 (DE3), and was purified as previously described (77).

678 The plasmid expressing GST-HsFascin1 was provided by Prof. David Kovar (University of
679 Chicago). The Homo sapiens Fascin1 full-length protein (Uniprot #Q16658) was produced in
680 BL21(DE3) competent bacterial cells from the pGEX-4T-3 vector as described previously with
681 some modifications (11). In brief, the bacterial cells were grown for 24 hours at RT (around
682 20°C) in auto-induction media (AIMLB0210, Formedium, UK), inoculated with overnight
683 growth starter culture. The cells were harvested by centrifugation and resuspended in PBS buffer
684 supplemented with 1 mM DTT, 10 mM CaCl₂, cComplete™ Protease Inhibitor Cocktail
685 (#11697498001, Merck), and DNaseI (Sigma, DN25-1G). The cells were disrupted by
686 sonication, centrifuged for 1-hour at 18,000 x g and the soluble fraction was then applied to a
687 Glutathione Sepharose 4 Fast Flow purification resin (#17513201, Cytiva). After 2 hours
688 incubation at 4°C, the beads were extensively washed with the same buffer and thrombin
689 protease was added to beads, thoroughly mixed, and incubated over night at 4°C. The next day
690 the cleaved soluble fraction from the beads was collected and subjected to a HiLoad 16/600
691 Superdex 75 gel filtration chromatography column, preequilibrated with 20 mM HEPES pH 8.0,
692 50 mM NaCl, 0.01% NaN₃. The elution fractions containing the protein were collected and
693 concentrated with 30 kDa MWCO VivaSpin columns. Fascin was labelled with the Alexa
694 Fluor™ 488 C 5 Maleimide (Invitrogen) with a 2x excess dye to protein molar ratio. Samples
695 were left to react overnight at 4°C under agitation. Excess dye was removed using a PD
696 MidiTrap G-25 column (Cytiva) in 20 mM HEPES, pH 8.0, 50 mM NaCl, 0.01% NaN₃. Aliquots
697 were flash-frozen in liquid nitrogen, and stored at -80°C until used for experiments.

698 **Actin co-sedimentation assay.** Non-muscle actin (#APHL-99, Cytoskeleton Inc.) at a final
699 concentration of 5 μ M was pre-polymerized in G-Buffer (2mM Tris pH 7.5, 0.2 mM ATP, 0.5
700 mM beta-mercaptoethanol, 0.2 mM CaCl₂) by adding 10x Initiation mix (200 mM HEPES pH
701 7.4, 1 M KCl, 1 mM EGTA, 10mM MgCl₂, 2 mM ATP) and incubating for 30 min at RT. Then,
702 recombinantly produced HsFascin was added at a final concentration of 1 μ M for 10 min at RT.
703 The mixture was then centrifuged at low speed (19,000 rpm, equivalent to around 14,000 x g),
704 which sediments only actin filament bundles. As controls, HsFascin alone, and filamentous actin
705 alone was tested side-by-side. At the end of the centrifugation, the supernatant fractions were
706 carefully separated from the pellets, Laemmli sample buffer was added, and fractions were
707 resolved on SDS-PAGE (#4561094, Biorad).

708 **GUV sample preparation and observation**

709 For all experiments, coverslips were passivated with a β -casein solution at a concentration of 5 g
710 L⁻¹ for at least 5 min at room temperature. Experimental chambers were assembled by placing a
711 silicon open chamber (2 well culture inserts, Ibidi) on a coverslip.

712 Actin polymerization buffer (F-buffer) contained 60 mM NaCl, 1 mM MgCl₂, 0.2 mM EGTA,
713 0.2 mM ATP, 10 mM DTT, 1 mM DABCO, 5 mM Tris pH 7.5. Actin polymerization buffer
714 without NaCl (F-buffer-no-NaCl) contained 1 mM MgCl₂, 0.2 mM EGTA, 0.2 mM ATP, 10 mM
715 DTT, 1 mM DABCO, 5 mM Tris pH 7.5.

716 GUVs were mixed sequentially with the ingredients to reach a total volume of 50 μ L, if present,
717 in the following order: NaCl (3 μ L from 1 M stock to reach a final concentration of 60 mM), F-
718 buffer-no-NaCl, IRSp53, VASP, fascin, profilin, capping protein, phalloidin, GUVs (20 μ L) and
719 finally actin. The GUV-protein mixture was then pipetted using a pipette tip with its tip cut into
720 the experimental chamber, followed by incubating at room temperature for at least 15 min before
721 observation. The final concentration of proteins, if present, were: 16 nM IRSp53, 4 nM VASP,
722 250 nM fascin, 0.6 μ M profilin, 25 nM capping protein and 0.5 μ M actin. If needed, we diluted
723 protein stocks in F-buffer so that the final concentrations of salt and ATP in the GUV-protein
724 mixtures was held approximately constant. To visualize actin by confocal microscopy,
725 depending on the experiments, we either used actin monomers having 10% - 27% fluorescently
726 labelled with AX488 or AX594, or unlabeled actin that included AX488- or AX594-conjugated
727 phalloidin (AX488 phalloidin or AX594 phalloidin) at a final concentration of 0.66 μ M.

728 Single actin filaments were prepared by mixing actin monomers with F-buffer to reach a final
729 actin concentration of 0.5 μ M.

730 Samples were observed using either spinning disk confocal microscopes or a laser scanning
731 confocal microscope. The two spinning disk confocal microscopes used were: (1) a Nikon
732 eclipse Ti-E equipped with a Yokogawa CSU-X1 confocal head, a 100X CFI Plan Apo VC
733 objective and a CMOS camera, (Prime 95B, Photometrics); and (2) a Nikon eclipse Ti-E
734 equipped with a 100x/1,4 OIL DIC N2 PL APO VC objective and an EMCCD camera (Evolve).
735 The laser scanning confocal microscope was a Nikon TE2000 microscope equipped with an C1
736 confocal system and a 60X water immersion objective (Nikon, CFI Plan Apo IR 60XWI ON
737 1.27 DT 0.17).

738 **Characterization of the orientational properties of actin-filled membrane tubes**

739 To characterize the orientational properties of GUVs with actin-filled membrane tubes, we
740 performed image analysis using the “OrientationJ” Plugin in ImageJ/Fiji (78). This plug-in
741 performs orientation analysis and provides a visual representation of the orientation of the image
742 and coherency of each pixel in the image that indicates if the local structural feature is oriented
743 or not. The coherency index C is between 0 and 1; $C = 1$ when the local structure has one
744 dominant orientation and $C = 0$ when the image is locally isotropic.

745 **Quantification of AX488 actin on GUV membranes**

746 Membrane fluorescence signals of GUVs were used to detect the contours of the GUVs by using
747 the “Fit Circle” function in ImageJ/Fiji. Then, a 5-pixel wide band centered on the GUV
748 contours was used to obtain the actin intensity profile of the band where the x-axis of the profile
749 is the length of the band and the y-axis is the averaged pixel intensity along the band width.
750 Following background subtraction, the actin intensity was then obtained by calculating the mean
751 value of the intensity values of the profile. To obtain the background intensity of AX488

752 fluorescence, we first manually drew a 5-pixel wide line perpendicularly across the GUV
 753 membranes (in which the x-axis of the profile is the length of the line and the y-axis is the
 754 averaged pixel intensity along the width of the line), and then the background intensity was
 755 obtained by calculating the mean value of the sum of the first 10 intensity values and the last 10
 756 intensity values of the background intensity profile.

757 **Estimation of the number of actin filaments in protrusions**

758 To estimate the number of actin filaments inside protrusions, we extracted fluorescence signals
 759 of actin in protrusions and normalized them by the fluorescence signals of single actin filaments
 760 in the bulk prepared by using the same actin stock as those in the GUV-protein mixture. The
 761 microscope settings for image acquisition were identical for the GUV sample and for the
 762 corresponding single actin filaments in the bulk. We performed the following steps to extract
 763 actin signals in protrusions and in the bulk. We manually defined the ROI, a line with a width of
 764 5 pixels drawn perpendicularly across protrusions or single actin filaments. We then obtained the
 765 actin fluorescent intensity profile of the line where the x-axis of the profile is the length of the
 766 line, and the y-axis is the averaged pixel intensity along the width of the line. The background
 767 intensity was obtained by calculating the mean value of the sum of the first five intensity values
 768 and the last five intensity values of the intensity profile. Finally, the actin fluorescence intensities
 769 were obtained by subtracting the background intensity from the maximum intensity value in the
 770 intensity profile. This image process was performed using ImageJ/Fiji (79).

771 **Characterization of IRSp53 clustering on GUVs**

772 To define clusters of IRSp53 on a surface of a GUV we segmented fluorescence images of the
 773 protein using a custom-made ImageJ/Fiji script based on the Rényi Entropy algorithm (80). We
 774 first created a mask of the fluorescent image in the IRSp53 channel using a Rényi Entropy
 775 threshold, then used the “Analyze Particles” function in ImageJ/Fiji to define clusters as a set of
 776 non-connected areas that have non-zero values in the mask image, permitting quantification of
 777 the number of clusters and their areas.

778 Coarse-grained Model and Clustering Analysis

779 The coarse-grained (CG) model used here has been discussed in detail previously (81, 82). The
 780 low-resolution, phenomenological CG model contains a 3-bead quasi-monolayer membrane
 781 model and a curved I-BAR domain model. The quasi-monolayer model is highly tunable and can
 782 accommodate significant remodeling, which makes it appealing for the application to I-BAR
 783 domains. The 3-beads interact internally with two harmonic bonds with a force constant of 25
 784 $k_B T$ and an equilibrium distance of 0.9 nm and a harmonic angle potential with a force constant
 785 of 10 $k_B T$ and an equilibrium angle of 180 degrees. The intermolecular forces were modeled
 786 using a soft pair potential, shown below, where A and B dictate the softness of the repulsion and
 787 the depth of the attraction and r_0 and r_c are the onset of repulsion cutoff and attraction cutoff,
 788 respectively.

$$789 \quad F_{pair}(r) = \begin{cases} A \cos\left(r \cdot \frac{\pi}{2r_0}\right), & \text{if } r \leq r_0 \\ B \cos\left(\frac{\pi}{2} + (r - r_c) \cdot \frac{\pi}{r_c - r_0}\right), & \text{if } r_0 < r \leq r_c \\ 0, & \text{if } r_c < r \end{cases}$$

790 Both the membrane and the protein have an A value of $25 k_B T$ to maintain an excluded volume
791 and a r_c value of $2 \cdot r_0$, where r_0 differs between the membrane and protein. Hence, r_0 and B
792 distinguish the species in the model. The membrane model is three linear beads: the two on the
793 ends are smaller and only interact with other lipids while the central membrane bead is larger and
794 attracted to other membrane beads as well as the membrane binding interface of the protein. The
795 smaller beads have a B value of 0 (i.e., no attraction), and a r_0 value of 1.125 nm. The central
796 membrane beads have a membrane-membrane B value of $0.6 k_B T$ and a r_0 value of 1.5 nm. The
797 PS-like and PIP₂-like membrane beads interact the same with each other, the key difference
798 being the protein-membrane interaction. With this parameter set, the quasi-monolayer is fluid
799 with a bending modulus around $10 k_B T$. The protein model is made of three curved strings of
800 beads to capture the curvature and size of the I-BAR domain of IRSp53 with a r_0 value of 1.7
801 nm. There are two outer strings of beads (shown in Red in Fig. 2D *Top* and Movie S2) that
802 capture the shape with a B value of 0 while the central string is the membrane binding interface.
803 The middle beads of the central string represent a weakly binding surface that is attracted to the
804 all-membrane beads with a B value of $0.235 k_B T$ (shown in Yellow in Fig. 2D *Bottom* and
805 Movie S3) and the ends of the membrane binding interface have a B value of $0.235 k_B T$ to PS-
806 like membrane beads and a B value of $0.705 k_B T$ to PIP₂-like membrane beads. The PIP₂-like
807 membrane beads are attracted to the ends of the I-BAR domain model to recapitulate the
808 experimentally observed behavior of PIP₂ clustering by I-BAR domains (31).

809 All systems were run using the LAMMPS molecular dynamics engine with a timestep of 200 fs
810 (83). The initial positions of the system were a flat membrane of 188,031 membrane beads with
811 65 I-BAR proteins slightly above the membrane. The systems were equilibrated for 50 million
812 timesteps in the NP_{XY}L_ZT ensemble at 0 surface tension using the Parrinello-Rahman barostat
813 with a dampening constant of 200 ps (91). Temperature was maintained using the Langevin
814 thermostat with a dampening constant of 20 ps (92). The simulations were then run for another
815 500 million timesteps in the NVT ensemble.

816 Clustering analysis was performed by creating a network of neighbors, where I-BAR domains
817 were neighbors if they were within 2 nm in the xy-plane. Two I-BAR domains were part of the
818 same cluster if and only if a path existed in the network of neighbors. Thus, any I-BAR domain
819 without a neighbor was considered free and not part of any aggregate. The probability density
820 was estimated using kernel density estimation from the histogram of neighbor sizes from the last
821 250 million timesteps of the simulation and subsequently used to calculate the probability of I-
822 BAR domain aggregates containing less than five I-BAR domains and five or more I-BAR
823 domains. The PIP₂-like membrane beads were separated into two groups: within 2 nm of an I-
824 BAR domain in the xy-plane (neighboring membrane beads) and not within 2 nm of an I-BAR
825 domain (bulk membrane beads). The percent of PIP₂-like membrane beads within each group
826 was quantified to measure the enrichment of PIP₂ near I-BAR domains. The CG model analysis
827 was performed using numpy, scikit-learn, and freud python packages and results plotted using
828 matplotlib python package (84-87). All snapshots of the CG systems were created using VMD
829 1.9.2 (88).

830 Live-cell imaging and membrane nanotube pulling experiments

831 **Cell culture, plasmids and transfection**

832 Rat2 fibroblasts (CRL-1764, ATCC) were cultured in DMEM GlutaMAX high-glucose media
833 (Gibco) supplemented with 1 mM sodium pyruvate (Gibco), 10% fetal bovine serum (Eurobio),

834 and 1% penicillin and streptomycin (Sigma-Aldrich). Cells were maintained in a humidified
835 incubator with 5% CO₂ at 37°C. Rat2 cells were routinely monitored for mycoplasma
836 contamination and found to be negative.

837 Common plasmids used for mammalian cell transfection include: (1) IRSp53 (human, full-
838 length, Uniprot #Q9UQB8) cloned into pmCherry-N1 and pEGFP-N1 vectors (Clontech) fusing
839 the fluorescent proteins to the C-terminus of IRSp53 (IRSp53-mCherry, IRSp53-eGFP); (2)
840 IRSp53's I-BAR domain (mouse, residues 1–250, Uniprot #Q8BKX1) cloned into a pEGFP-N1
841 vector (I-BAR-eGFP); (3) Fascin 1 (human, full-length, Uniprot #Q16658) cloned into
842 pmCherry-C1 and pEGFP-C1 vectors (Clontech) fusing the fluorescent proteins to fascin's N-
843 terminus (mCherry-fascin and eGFP-fascin); (4) pmTagRFP-VASP (mouse, full-length,
844 Uniprot# P70460) which was a gift from Michael Davidson (Addgene plasmid #58027); and (5)
845 an empty mCherry vector.

846 For the live-cell imaging experiments (Fig. 1 and Fig. 4), cells were transiently transfected the
847 day before imaging using the XfectTM transfection reagent (Takara Bio) according to the
848 manufacturer's recommendations and replated on fibronectin-coated glass-bottom dishes (35
849 mm, No. 1.5H, Ibidi) two hours prior to imaging. Fibronectin (from human plasma, >95%,
850 Sigma) coating was done by incubating the dishes 1 hour at RT (or overnight at 4°C) with a 10
851 µg/mL fibronectin solution dissolved in PBS.

852 For the nanotube pulling experiments (Fig. 5 and Fig. 6), stable Rat2 cell lines expressing
853 IRSp53-eGFP or I-BAR-eGFP were generated by antibiotic selection (0.5 mg/mL geneticin
854 (G418), 7 days) post transfection (FuGENE HD, Promega) followed by cell sorting using a
855 SH800 cell sorter (Sony Biotechnology) to collect a pool of fluorescent positive cells. For Fig.
856 S19, wild-type Rat2 cells were transiently transfected using FuGENE HD (Promega) or XfectTM
857 (Takara Bio) reagents to achieve double-expressing IRSp53-eGFP/RFP-VASP or eGFP-
858 Fascin/IRSp53-mCherry cells.

859 **Live-cell imaging of filopodia formation**

860 Time-lapse image series for double-transfected Rat2 cells expressing IRSp53-eGFP/RFP-VASP,
861 IRSp53-eGFP/mCherry-fascin or IRSp53-eGFP/mCherry were obtained using a GE DeltaVision
862 Ultra Widefield microscope equipped with a pco.edge 4.2ge sCMOS camera (PCO GmbH),
863 Solid State Illuminators (SSI) for fluorescence excitation, an incubation system set at 37°C and
864 5% CO₂ and 63x or 100x oil objectives. Deconvolution of acquired time-lapse series was
865 performed using the built-in microscopy software (softWoRxTM). Further analysis was conducted
866 with the Microscopy Image Browser (MIB), a free MATLAB-based software developed by Ilya
867 Belevich (University of Helsinki).

868 **Filopodia quantification**

869 Each analyzed filopodia was monitored from the initiation of membrane bending (cone
870 formation) until the beginning of active retraction. Changes in spatial coordinates of the positions
871 of base and tip with the times were used to calculate the length of growing filopodia
872 ("Distance"). The rate of growth (µm/sec) was calculated as the first derivative of filopodia
873 length with respect to time ($\frac{\partial(Distance)}{\partial(Time)}$). Changes in the rate of growth were used to assess
874 peculiarities of filopodia formation (stops, i.e., when $\frac{\partial(Distance)}{\partial(Time)} = 0$, and instantaneous

875 retractions when $\frac{\partial(\text{Distance})}{\partial(\text{Time})} < 0$). The frequency of each event (sec^{-1}) was calculated as the
876 number of stops or retractions per second registered for the period of active filopodia growth.

877 To assess the effect of fascin accumulation in filopodia on their elongation rates, separate
878 kymographs of growing filopodia (time-space plots) for IRSp53-eGFP and mCherry-fascin
879 channels were generated using the “Multi-Kymograph” plug-in
880 (<https://www.embl.de/eamnet/html/kymograph.html>) in ImageJ/Fiji. Velocities were measured in
881 ImageJ/Fiji for selected linear intervals of the kymographs (about 10-20 sec) before and after
882 fascin appearance using the “Velocity Measurement Tool” macro
883 (<https://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Velocit%20Measurement%20Tool>).

884 We followed the colocalization analysis as published previously (46) using the “Colocalization
885 colormap” (89) plug-in implemented in ImageJ/Fiji. For each pixel in an image, one calculates
886 the deviation of the fluorescent intensity from the mean intensity of the whole image. Then, one
887 calculates the mean deviation product of the two protein signals, $(I_{IRSp53} - \bar{I}_{IRSp53})(I_{VASP} - \bar{I}_{VASP})$,
888 where I and \bar{I} are the pixel intensity and the mean intensity of the image, respectively.
889 Finally, to compare series of images, one calculates the normalized mean deviation product
890 named nMDP, $(I_{IRSp53} - \bar{I}_{IRSp53})(I_{VASP} - \bar{I}_{VASP}) / ((I_{IRSp53}^{max} - \bar{I}_{IRSp53})(I_{VASP}^{max} - \bar{I}_{VASP}))$, where
891 I^{max} is the maximum intensity of the corresponding image. A spatial representation of the
892 correlation of the two protein signals can be obtained. We manually tracked filopodia and for
893 each time point, we averaged the nMDP values of four pixels located at the filopodial structures.
894 We overlapped the nMDP values, termed the colocalization index, during filopodia formation.

895 **Bead functionalization**

896 An aliquot of streptavidin-coated polystyrene beads (SVP-30-5, 0.5% w/v, Spherotech) having a
897 nominal diameter of 3 μm was washed three times in a 10X volume of PBS. Between washing
898 steps, beads were pelleted using a centrifuge at 12,000 rpm for 5 min. The final pellet was
899 resuspended in PBS to a concentration of 0.05% w/v. Next, a volume of biotin-conjugated
900 concanavalin A (ConA; C2272, Sigma-Aldrich), having a stock concentration of 1 mg mL^{-1} in
901 PBS, was added to the bead suspension assuming a binding capacity of 10 μg protein per mg of
902 solid particles. The mixture was incubated overnight at 4°C on a tabletop shaker set to 1,500
903 rpm. The ConA-coated beads were rinsed three times according to the steps above and finally
904 resuspended in PBS to a concentration of 0.5% w/v. ConA-beads were stored at 4°C and
905 generally usable up to one month.

906 **Nanotube pulling experiments**

907 A custom-built optical tweezer setup coupled to an inverted Nikon C1 Plus laser scanning
908 confocal microscope, as previously described in (61), was used for pulling plasma membrane
909 nanotubes, force measurements, and simultaneous fluorescence imaging. Briefly, a 1064 nm
910 continuous wave Ytterbium fiber laser (IPG Photonics) set to a 3 W input power was modulated
911 to 400 mW (measured at the back aperture of the objective) using a polarizing beam splitter
912 (Thorlabs), expanded through a telescope consisting of two plano-convex lenses with focal
913 lengths of 100 mm and 150 mm (Thorlabs), and directed towards the back aperture of a Nikon
914 CFI Plan Apochromat Lambda 100X 1.45 NA oil immersion objective. The trap stiffness κ was
915 determined using the viscous drag method, including Faxen’s correction for calibration near
916 surfaces (90) and averaged 60 pN μm^{-1} . Displacements of a trapped bead from the fixed trap
917 center were recorded using a Marlin F-046B CCD camera (Allied-Vision) at a frame rate of 20

918 frames per second, and later analyzed by a custom MATLAB (Mathworks) script utilizing the
919 *imfindcircles* function to output the center location of the tracked bead (in μm). Forces were
920 calculated from the determined bead positions according to the equation, $F = \kappa \cdot (x - x_o)$,
921 where κ is the trap stiffness, x is the displaced bead position, and x_o is the equilibrium reference
922 position of the trapped bead. As the optical trap itself was stationary, all relative movements
923 were performed using a piezo-driven stage (Nano-LP100, MadCityLabs). Atop the stage, a
924 temperature and CO_2 controllable stage-top incubator (STXG-WELSX, Tokai Hit) was attached,
925 allowing cells to be maintained at 37°C in a humidified, 5% CO_2 atmosphere during
926 experimentation.

927 The day before, Rat2 cells expressing I-BAR-eGFP or IRSp53-eGFP were plated on fibronectin-
928 coated ($35 \mu\text{g mL}^{-1}$) glass bottom dishes (35 mm, No. 1.5, MatTek) at a density of $\sim 30,000$ cells
929 cm^{-2} . Thirty to sixty minutes prior to experimentation the phenol-containing culture medium was
930 removed, cells were rinsed with PBS, and phenol-free DMEM containing ProLongTM Live
931 Antifade Reagent (Invitrogen) at a 1:75 dilution, and 2 mg mL^{-1} β -Casein ($>98\%$ pure, from
932 bovine milk, C6905, Sigma-Aldrich), for surface passivation, was applied. The cells were taken
933 to the optical tweezer setup and labeled with Cell MaskTM Deep Red plasma membrane stain
934 (Invitrogen) at a 1:2000 dilution for 10 minutes, and ConA-coated beads were added (1:50–1:100
935 dilution). Using a custom LabVIEW (National Instruments) program to control the piezo stage,
936 membrane nanotubes were pulled by trapping an isolated floating bead, bringing it into contact
937 with the cell for a short period of time (<10 sec), and then moving the cell away from the bead in
938 the x direction.

939 Confocal images were acquired using solid-state excitation lasers: a 488 (Coherent), 561
940 (Coherent) and a 642 nm (Melles Griot). The detection pathway consisted of a T560lpxr dichroic
941 beamsplitter (Chroma), an ET525/50 bandpass filter (Chroma), an ET665 longpass filter
942 (Chroma), and two τ -SPAD single-photon avalanche diodes (PicoQuant). The τ -SPADs were
943 controlled by the SymPhoTime 64 software (PicoQuant). Images encompassing the nanotube and
944 some of the cell body (typically 1024×512 pixels, 5X zoom) were gathered 5 minutes after the
945 nanotube was pulled for protein sorting analysis; identical acquisition parameters were used
946 when gathering the membrane and protein channel data for an individual nanotube.

947 **Sorting analysis**

948 Image analysis was performed using custom-written macros in the ImageJ/Fiji software. To
949 quantify protein enrichment in the nanotube (t) relative to the bulk plasma membrane of the cell
950 (ref), the sorting parameter (S) is defined as $S = \frac{I_t^P / I_{ref}^P}{I_t^L / I_{ref}^L}$ where the ratio of the green protein
951 fluorescence (I^P) in the nanotube and in the cell is normalized by the same ratio of the red lipid
952 fluorescence (I^L). This is to correct for cell-to-cell differences in both the protein and membrane
953 signal intensity (e.g., protein expression levels, different acquisition parameters between cells,
954 efficiency of membrane staining). Sorting values of $S > 1$ signify the protein is enriched in the
955 nanotube while $S < 1$ signify the protein is excluded from the nanotube, with respect to its
956 average density in the cell plasma membrane. Given Rat2 fibroblasts are quite flat, we focused
957 on the ventral side of the cell to obtain reference images of the plasma membrane for both the
958 protein and membrane channels. Images were background subtracted and common regions of
959 interest ($\sim 20,000$ pixels in size) were then manually drawn to encompass homogeneous areas of
960 the plasma membrane (near the site of the nanotube and excluding areas where vesicular puncta

961 were observed) to calculate average protein and lipid reference values, I_{ref}^P and I_{ref}^L ,
962 respectively. The protein and membrane channels of the in-focus nanotube were also subjected to
963 background subtraction and then normalized by their respective reference values to generate a
964 heat map of S values. The resulting S map was filtered by a 3x3 adaptive median filter
965 (<https://weisongzhao.github.io/AdaptiveMedian.imagej/>) to remove spurious pixels in the
966 background; this processing was done given the pixel values of the raw images are discrete
967 values (photon counts, cts) and not continuous values. The width of the nanotube was fit to a
968 Gaussian and a rectangular region of interest (ROI; size $\pm 2\sigma$ of the Gaussian profile) was defined
969 along the length of the nanotube in the S map. Orthogonal cross sections were iteratively
970 generated pixel-by-pixel along the length of the nanotube within the ROI. The maximum S value
971 for each cross section was determined and then averaged to report the mean sorting value of the
972 protein in the nanotube (S_{avg}).

973 **Statistical analysis**

974 All graphs and statistical analyses were performed using the GraphPad Prism software (versions
975 9.3.1 or 9.4.0 for Mac or Windows, respectively). p values < 0.05 were considered statistically
976 significant and all p values are indicated in the figures. The specific statistical tests performed,
977 the number of independent experiments and the total number of samples analyzed are indicated
978 in the figure captions.

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1231

1232 Acknowledgments

1233 The computations were supported by the University of Chicago Research Computing Center
1234 (RCC). We thank Evelyne Coudrier and Camille Simon for insightful discussions. We also thank

1235 Fahima Di Federico for handling plasmids, Fanny Tabarin-Cayrac for cell sorting and Anne-
1236 Sophie Mace for ImageJ programming assistance. F-CT, CLC and PB are members of the CNRS
1237 consortium AQV. F-CT and PB are members of the Labex Cell(n)Scale (ANR-11-LABX0038)
1238 and Paris Sciences et Lettres (ANR-10-IDEX-0001-02). The authors greatly acknowledge the
1239 Cell and Tissue Imaging core facility (PICT IBiSA), Institut Curie, member of the French
1240 National Research Infrastructure France-BioImaging (ANR10-INBS-04).

1241 **Funding:**

1242 Human Frontier Science Program (HFSP) grant RGP0005/2016 (F-CT, JMH, GAV, PL and PB)
1243 Institut Curie and the Centre National de la Recherche Scientifique (CNRS) (F-CT, JMH, and
1244 PB)

1245 Marie Curie actions H2020-MSCA-IF-2014 (F-CT)

1246 EMBO Long-Term fellowship ALTF 1527-2014 (F-CT)

1247 Pasteur Foundation Fellowship (JMH)

1248 Agence Nationale pour la Recherche ANR-20-CE13-0032 (JMH and PB)

1249 Université Paris Sciences et Lettres-QLife Institute ANR-17-CONV-0005 Q-LIFE (PB)

1250 FY 2015 Researcher Exchange Program between the Japan Society for the Promotion of Science
1251 and Academy of Finland (YS)

1252 Takeda Science Foundation (YS)

1253 Wesco Scientific Promotion Foundation (YS)

1254 Agence Nationale pour la Recherche ANR-18-CE13-0026-01 and ANR-21-CE13-0010-03
1255 (CLC)

1256 Cancer Society Finland 4705949 (PL)

1257 United States National Institutes of Health (NIH) Institute of General Medical Sciences
1258 (NIGMS) grant R01-GM063796 (GAV and ZJ)

1259

1260 **Author contributions:**

1261 GAV, PL and PB designed the initial project. F-CT, JMH, ZJ, EK, YS, JP, OM, JM performed
1262 experiments and analyzed results under the supervision of CLC, GAV, PL and PB. F-CT and JP
1263 developed GUV experiments, and F-CT performed and analyzed data from the GUV
1264 experiments. JMH, ZJ, EK and JP performed and analyzed data from nanotube pulling
1265 experiments, computer simulations, live-cell imaging experiments, and actin pyrene assays,
1266 respectively. YS, JP and JM purified proteins. OM analyzed protein clustering on GUVs. F-CT
1267 wrote the original draft with inputs and revisions from JMH and PB.

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1276 Funding acquisition: GAV, PL, PB

1277

1278 **Competing interests:** The authors declare that they have no competing interests.

1279

1280 **Data and materials availability:** All data needed to evaluate the conclusions in the paper are
1281 present in the paper and/or the Supplementary Materials.

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Figures and Tables

1301 **Fig 1. Dynamics of VASP clusters assembled from preexisting IRSp53 clusters on the**
1302 **plasma membrane in filopodia initiation.**

1303 (A) Widefield fluorescence image of a representative Rat2 cell transfected with IRSp53-eGFP
1304 and RFP-VASP. Brackets indicate some filopodia where IRSp53 is present along them. White
1305 arrows indicate the same filopodia to demonstrate that VASP is enriched in their tips. Scale bar,
1306 5 μm . (B) Time-lapse images of a filopodium formation. Images are magnifications of the
1307 indicated area (cyan boxes) in (A). The white arrow indicates the appearance of an IRSp53
1308 cluster followed by a VASP cluster indicated by a cyan arrow at the onset of filopodia formation.
1309 White boxes indicate the selected area used to generate outlines of plasma membrane positions
1310 over time shown in (C). Scale bar, 2 μm . (C) Colored outlines of membrane positions in the
1311 region indicated by the white boxes shown in (B). Total 27 frames, frame interval 2 sec. (D)
1312 Adaptive kymograph maps replot the detected membrane profiles in (C) in the y-axis and the
1313 corresponding time points in the x-axis to show the dynamics of IRSp53 (*Left*) and VASP
1314 (*Right*) on the plasma membrane over time. Y-axis shows the membrane positions of the
1315 proteins, and the x-axis shows the time (in second, total 27 frames). Y-axis scale bars, 1 μm .
1316 Color maps: low fluorescence intensity in blue, and high fluorescence intensity in red. Circled
1317 numbers correspond to the frames indicated in (B).

1318 **Fig. 2 IRSp53 self-assembles into clusters and recruits VASP on PIP₂-membranes.**

1319 (A) and (B) Representative confocal images of GUVs incubated with AX488-labeled IRSp53 (16
1320 nM). GUVs contain TBX with 0.5% TR-ceramide and either 5% PIP₂ (PIP₂-GUVs) in (A) or
1321 25% DOPS (PS-GUVs) in (B). TR-ceramide in magenta and IRSp53 in cyan. Arrows indicate
1322 IRSp53 clusters on GUV membranes. Arrowhead indicates an inward membrane tube generated
1323 by IRSp53. (C) Sizes of IRSp53 clusters on PIP₂-GUVs and on PS-GUVs. Each data point
1324 represents one cluster. PIP₂-GUV: total 225 clusters, N = 42 GUVs, 3 sample preparations. PS-

1325 GUV: total 55 clusters, $N = 12$ GUVs, 2 sample preparations. Statistical analysis: two-tailed
1326 Mann-Whitney test, $p = 0.1125$. (D) Top: Representative snapshots of coarse-grained (CG)
1327 simulations from PS-like (0% PIP₂), 2% PIP₂-like, and 5% PIP₂-like membranes. Membrane CG
1328 beads in grey, PIP₂-like CG beads in blue, and I-BAR domains in red. Scale bar, 50 nm. Bottom:
1329 Enlarged areas as indicated by the white boxes. Only the central portion of the I-BAR domain is
1330 shown (yellow) to visualize PIP₂ clusters (blue). (E) Probability of I-BAR domain aggregate size
1331 to be < 5 or ≥ 5 molecules for membranes shown in (D). (F) Representative confocal images of
1332 GUVs incubated with AX488-labeled VASP (yellow) together with (*Top*) or without (*Bottom*)
1333 IRSp53 (unlabeled). Protein concentrations: IRSp53, 16 nM; VASP, 4 nM. GUVs contain TBX
1334 with 0.5% TR-ceramide (magenta) and 5% PIP₂. Heat maps in (A) and (B) for IRSp53 signals,
1335 and (F) for VASP signals; low fluorescence intensity in blue, and high intensity in red. Scale
1336 bars, 5 μm .
1337

1338 **Fig. 3. IRSp53 and VASP synergistically drive the formation of actin-filled membrane**
1339 **protrusions.**

1340 (A-D) Representative GUVs (membranes, magenta; actin, green) incubated with: (A) all proteins
1341 and (B) where actin was labelled instead with AX488 phalloidin; and where (C) IRSp53 and (D)
1342 VASP were excluded. Cartoon in (A) depicts GUVs incubated with all proteins (CP and profilin
1343 not shown). All proteins: IRSp53 (16 nM), VASP (4 nM), actin (0.5 μ M, 10% - 27% AX488
1344 labelled), fascin (250 nM), CP (25 nM) and profilin (0.6 μ M). GUV composition: TBX, 0.5%
1345 TR-ceramide and 5% PIP₂. Scale bars, 5 μ m. (D) Right: pixel-averaged actin signals on GUVs.
1346 “No VASP” N = 29 GUVs, “With VASP” N = 29, 1 preparation (see Fig. S12A for another two
1347 preparations). (E) Percentages of GUVs having actin-filled tubes with (“With VASP”) and
1348 without (“No VASP”) VASP. “With VASP” N = 56, 39, 45; “No VASP” N = 42, 41, 33; 3
1349 preparations. Statistical analyses: chi-squared test on pooled data, $p < 0.0001$; paired t test, $p =$
1350 0.0585 . (F, G) Percentages of tube-positive GUVs in the absence (“No Actin”) and presence
1351 (“With Actin”) of actin. “With Actin” GUVs were counted only when having actin-filled tubes
1352 (*). (F) “No phalloidin” corresponds to AX488-labelled actin. “No Actin” N = 39, 60; “With
1353 Actin” N = 45, 56; 2 preparations. Statistical analyses: chi-squared test on pooled data, $p <$
1354 0.0001 ; paired t test, $p = 0.3072$. (G) In the presence of AX488 phalloidin, and no AX488-
1355 labelled actin. “No Actin” N = 31, 31, 26, 39; “With Actin” N = 54, 57, 41, 56; 4 preparations.
1356 Statistical analyses: chi-squared test on pooled data, $p < 0.0001$; paired t test, $p = 0.0054$.

1357 **Fig. 4. Fascin facilitates filopodia growth and prevents filopodia retraction.**

1358 (A) Left: Representative images of GUVs incubated with all protein ingredients (IRSp53, VASP,
1359 actin, CP and profilin) besides Fascin. Right: Percentages of GUVs having actin-filled tubes in
1360 the absence (“No Fascin”) and presence (“With Fascin”) of Fascin. GUV composition: TBX,
1361 0.5% TR-ceramide and 5% PIP₂. “No Fascin” N = 53, 32, 54 GUVs; “With Fascin” N = 56, 45,
1362 39; 3 sample preparations. Statistical analyses: chi-squared test on pooled data, $p = 0.8652$;
1363 paired t test, $p = 0.6906$. (B) Widefield fluorescence image of a Rat2 cell transfected with
1364 IRSp53-eGFP and mCherry-fascin. (C) Time-lapse images of the formation of a filopodium.
1365 Images are magnifications of the indicated area (white boxes) in (B). White arrows indicate the
1366 appearance of IRSp53 clusters followed by fascin recruitment. Time in sec. (D) Left:
1367 Representative kymograph of IRSp53 and fascin fluorescence signals in a growing filopodium
1368 showing the growth before (Number 1) and after (Number 2) the presence of fascin. Right:
1369 Quantification of filopodial growth rate before (“Before Fascin arrived”) and after (“After Fascin
1370 arrived”) fascin recruitment. N = 26 filopodia. Statistical analysis: Mann-Whitney nonparametric
1371 test, $p = 0.00001633$. (E) Frequency of filopodia retractions in IRSp53-eGFP expressing Rat2
1372 cells transfected with either an empty mCherry plasmid (“Control”) or mCherry-fascin
1373 (“Fascin”). The frequency of each event was calculated for the period of filopodia growth as the
1374 number of retractions per sec. “Control” N = 13 filopodia; “Fascin” N = 30. Statistical analysis:
1375 Mann-Whitney nonparametric test, $p = 0.000083802$. Scale bars: (A) 5 μ m; (B, C) 2 μ m.
1376

1377 **Fig. 5. IRSp53’s I-BAR domain is robustly recruited into pulled membrane nanotubes.**

1378 (A) Experimental setup for pulling membrane nanotubes using a concanavalin A (con-A)-coated
1379 bead trapped in an optical tweezer (OT). Rat2 fibroblasts, expressing eGFP fusions (green) of
1380 either IRSp53’s I-BAR domain or the full length (FL) IRSp53 protein, were labelled with Cell
1381 MaskTM Deep Red plasma membrane stain (magenta); protein enrichment in the membrane

1382 nanotube was monitored by confocal fluorescence microscopy using single-photon avalanche
1383 detectors (cts, counts). (B) Representative confocal image of a pulled membrane nanotube from a
1384 Rat2 cell expressing IRSp53's I-BAR domain showing high enrichment of the I-BAR domain.
1385 (C) Top: Calculated sorting map of the nanotube in (B) with low sorting (S) values in blue and
1386 high S values in red. Bottom: Plot of the maximum sorting value at each pixel position along the
1387 length of the nanotube (white bracket in the sorting map) and the mean sorting value for the
1388 protein (S_{avg}). (D) Measured S_{avg} values for IRSp53's I-BAR domain in pulled nanotubes ($N = 19$
1389 nanotubes). $S_{\text{avg}} > 1$ (dashed black line) indicate protein enrichment. Black solid line, mean of
1390 the data points. Dashed white circles in the figure outline the trapped bead. Scale bars, $5 \mu\text{m}$.

1391 **Fig. 6. IRSp53 is recruited into membrane nanotubes pulled from highly active cellular**
1392 **zones and coincides with actin filament assembly.**

1393 (A) and (B) Representative confocal images of pulled membrane nanotubes from Rat2 cells
1394 expressing the full-length IRSp53 protein (IRSp53-eGFP). (A) Pulled nanotubes near cellular
1395 zones of active membrane remodeling, such as membrane ruffling (white arrowheads), exhibit
1396 recruitment of IRSp53 in the nanotube. (B) Pulled nanotubes near non-active zones instead show
1397 no recruitment of IRSp53. (C) Mean sorting values for a given nanotube, S_{avg} , were determined
1398 from active and non-active zones for IRSp53 expressing cells. Dashed black line, $S_{\text{avg}} = 1$. Black
1399 solid lines, mean of the data points. Active zone, $N = 13$ nanotubes; Non-active zone, $N = 20$
1400 nanotubes. (D) IRSp53 enrichment corresponds with eventual actin development within the
1401 pulled nanotube. Representative sorting (S) map (left) and the corresponding force plot (center)
1402 for a nanotube showing high IRSp53 sorting. Peaks in the force plot (black arrowheads) are
1403 signatures of actin in the tube, and arise when retrograde flows outcompete actin polymerization
1404 (at the nanotube tip) causing bead displacement towards the cell body and hence a rise in the
1405 force (right, i and ii). (E) Distribution of the force peak magnitudes (ΔF). Sample size, 100
1406 peaks. (F) Representative sorting map (left) and the corresponding force plot (right) for a
1407 nanotube showing no IRSp53 enrichment and hence no actin development. Color maps: low S
1408 values in blue, and high S values in red. Dashed white circles in the figure outline the trapped
1409 bead. Scale bars, $5 \mu\text{m}$.

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