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

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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 PIP<sub>2</sub>. 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

79 IRSp53 (also known as BAIAP2), an inverse BAR (I-BAR, Bin-Amphiphysin-  
80 Rvs161/167) domain protein, is a crucial player in coordinating actin assembly and membrane  
81 dynamics in filopodia formation (16-23). IRSp53 features an N-terminal I-BAR domain (also  
82 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)P<sub>2</sub> (hereafter as PIP<sub>2</sub>) (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 PIP<sub>2</sub> 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 PIP<sub>2</sub>-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 PIP<sub>2</sub> 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 PIP<sub>2</sub>-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 PIP<sub>2</sub>, 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 PIP<sub>2</sub>-  
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 PIP<sub>2</sub> in IRSp53 clustering, we tested if IRSp53 could form clusters  
201 upon interacting with another negatively charged lipid, PS, given that IRSp53-PIP<sub>2</sub> binding is  
202 driven by electrostatic interactions (25). We replaced PIP<sub>2</sub> 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 PIP<sub>2</sub>-  
204 GUVs (5 mole% of PIP<sub>2</sub>. At pH 7, the charge of PIP<sub>2</sub> 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 PIP<sub>2</sub>-GUVs and PS-GUVs (Fig. S3). However, the clusters are larger on  
207 PIP<sub>2</sub>-GUVs as compared to PS-GUVs (Fig. 2C). To elucidate the mechanism underlying  
208 IRSp53-clustering on PIP<sub>2</sub>-membranes, we performed coarse-grained (CG) simulations. We  
209 generated PS-like and PIP<sub>2</sub>-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 PIP<sub>2</sub>-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 PIP<sub>2</sub>-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% PIP<sub>2</sub>-like membrane) and the PIP<sub>2</sub>-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 PIP<sub>2</sub>-like membrane) and PIP<sub>2</sub>-like membranes (2%  
221 and 5% of PIP<sub>2</sub>-like membranes, Fig. 2D, Top). Moreover, we found that the addition of PIP<sub>2</sub>-  
222 like membrane beads increases the aggregation of I-BAR domains. Without PIP<sub>2</sub>-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 PIP<sub>2</sub>-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 PIP<sub>2</sub> microdomains upon membrane binding (31, 49). Indeed, we  
228 found an enrichment of PIP<sub>2</sub>-like membrane beads near I-BAR domain aggregates (Fig. 2D,  
229 Bottom, blue dots, and Movie S3). In the 2% PIP<sub>2</sub>-like membranes, we found 27% of  
230 neighboring membrane beads are PIP<sub>2</sub>-like, which is an approximate 14 times enrichment of  
231 PIP<sub>2</sub>-like membrane beads around the I-BAR domain aggregates compared to the total 2%  
232 concentration of PIP<sub>2</sub>-like beads on the membrane. Similarly, in the 5% PIP<sub>2</sub>-like membranes,  
233 we found 33% of neighboring membrane beads are PIP<sub>2</sub>-like, which is a 6.7 times enrichment of  
234 PIP<sub>2</sub>-like membrane beads to the aggregates compared to the total 5% concentration of PIP<sub>2</sub>-like  
235 membrane beads. The enrichment of PIP<sub>2</sub>-like membrane beads around I-BAR domain  
236 aggregates depletes PIP<sub>2</sub> in the bulk membrane that is not adjacent to an I-BAR domain  
237 aggregate. In other words, the PIP<sub>2</sub> 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 PIP<sub>2</sub>-like membrane beads for 2% and 5% PIP<sub>2</sub>-like membrane systems, respectively.  
240 Our results thus indicate positive feedback of the assembly of I-BAR domain aggregates  
241 mediated by PIP<sub>2</sub> and the enrichment of PIP<sub>2</sub> around the aggregates facilitate further I-BAR  
242 domain recruitment. Altogether, our simulation and experimental reconstitution results indicate  
243 the key role of PIP<sub>2</sub> in IRSp53 clustering on membranes.

244 It was reported that in bulk, as well as on small vesicles containing PIP<sub>2</sub>, 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 PIP<sub>2</sub>-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 PIP<sub>2</sub>-membranes generates actin-filled**  
262 **membrane protrusions**

263 We next assessed if IRSp53, VASP, actin and fascin can self-organize on PIP<sub>2</sub>-  
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 PIP<sub>2</sub>-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 PIP<sub>2</sub>-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 PIP<sub>2</sub>-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 PIP<sub>2</sub>-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 Mask<sup>TM</sup> 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 Mask<sup>TM</sup> 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 ( $S_{\text{avg}}$ ) (Fig. 5C; see Materials and Methods for further details). Note  
438 that  $S_{\text{avg}}$  values greater than 1 indicate preferential protein enrichment for a given nanotube. We  
439 measured the  $S_{\text{avg}}$  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  $\Delta F$ ) 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 PIP<sub>2</sub> 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 PIP<sub>2</sub>-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 PIP<sub>2</sub> is key for the assembly of these IRSp53 clusters: higher PIP<sub>2</sub>  
524 concentration results in larger I-BAR domain clusters. Importantly, our simulation results  
525 showed an at least 7-fold enrichment of PIP<sub>2</sub> 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 PIP<sub>2</sub>  
527 clusters (31, 49, 65). Notably, it was proposed that these PIP<sub>2</sub> clusters are transiently interacting  
528 with BAR domains such that PIP<sub>2</sub> in the clusters is available for the recruitment of downstream  
529 partners having PIP<sub>2</sub> binding motifs (31, 49, 65). We thus propose that besides specific protein-  
530 protein interactions, IRSp53-induced PIP<sub>2</sub> 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 PIP<sub>2</sub>-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 MgCl<sub>2</sub>, 0.2 mM EGTA, 0.2 mM ATP, 10 mM DTT, 1 mM DABCO, 5 mM  
602 Tris pH 7.5 and 0.01% NaN<sub>3</sub> in the presence of actin (2  $\mu$ M, 5% pyrenyl labeled), profilin (2.4  
603  $\mu$ M), VASP (15 nM in tetramer), CP (25 nM) and phalloidin (2  $\mu$ 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  $\mu$ M of actin, i.e., the total concentration of actin subtracts 0.1  $\mu$ 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- $\alpha$ -phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>,  
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.  $\beta$ -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)P<sub>2</sub> 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 ddH<sub>2</sub>O twice. The PVA-  
634 coated coverslips were incubated at 50°C for 30 min. Around 5  $\mu$ 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  $\mu$ 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 CaCl<sub>2</sub>, 0.2 mM ATP, 1 mM DTT, 0.01%  
645 NaN<sub>3</sub>) 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  $\mu$ g/mL ampicillin, and cultured at 37°C overnight. Then, the starter  
654 culture was inoculated into 1 L LB medium containing 100  $\mu$ g/mL ampicillin, and cultured at  
655 37°C until OD<sub>600</sub> 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  $\mu$ 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 CaCl<sub>2</sub>, 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% NaN<sub>3</sub>. 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% NaN<sub>3</sub>. 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 $\mu$ 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 CaCl<sub>2</sub>) by adding 10x Initiation mix (200 mM HEPES pH  
701 7.4, 1 M KCl, 1 mM EGTA, 10mM MgCl<sub>2</sub>, 2 mM ATP) and incubating for 30 min at RT. Then,  
702 recombinantly produced HsFascin was added at a final concentration of 1  $\mu$ 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  $\beta$ -casein solution at a concentration of 5 g  
710 L<sup>-1</sup> 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 MgCl<sub>2</sub>, 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 MgCl<sub>2</sub>, 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  $\mu$ L, if present,  
717 in the following order: NaCl (3  $\mu$ 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  $\mu$ 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  $\mu$ M profilin, 25 nM capping protein and 0.5  $\mu$ 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  $\mu$ M.

728 Single actin filaments were prepared by mixing actin monomers with F-buffer to reach a final  
729 actin concentration of 0.5  $\mu$ 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 PIP<sub>2</sub>-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 PIP<sub>2</sub>-like membrane beads. The PIP<sub>2</sub>-like  
807 membrane beads are attracted to the ends of the I-BAR domain model to recapitulate the  
808 experimentally observed behavior of PIP<sub>2</sub> 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<sub>XY</sub>L<sub>Z</sub>T 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 PIP<sub>2</sub>-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 PIP<sub>2</sub>-like membrane beads within each group  
826 was quantified to measure the enrichment of PIP<sub>2</sub> 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% CO<sub>2</sub> 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 Xfect<sup>TM</sup> 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 Xfect<sup>TM</sup>  
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% CO<sub>2</sub> and 63x or 100x oil objectives. Deconvolution of acquired time-lapse series was  
865 performed using the built-in microscopy software (softWoRx<sup>TM</sup>). 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 ( $\frac{\partial(\text{Distance})}{\partial(\text{Time})}$ ). Changes in the rate of growth were used to assess  
874 peculiarities of filopodia formation (stops, i.e., when  $\frac{\partial(\text{Distance})}{\partial(\text{Time})} = 0$ , and instantaneous

875 retractions when  $\frac{\partial(\text{Distance})}{\partial(\text{Time})} < 0$ ). The frequency of each event ( $\text{sec}^{-1}$ ) 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,  $(I_{IRSp53} - \bar{I}_{IRSp53})(I_{VASP} - \bar{I}_{VASP}) / ((I_{IRSp53}^{max} - \bar{I}_{IRSp53})(I_{VASP}^{max} - \bar{I}_{VASP}))$ ,  
891 where  $I^{max}$  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  $\mu\text{m}$  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  $\text{mL}^{-1}$  in  
901 PBS, was added to the bead suspension assuming a binding capacity of 10  $\mu\text{g}$  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  $\kappa$  was  
915 determined using the viscous drag method, including Faxen’s correction for calibration near  
916 surfaces (90) and averaged 60 pN  $\mu\text{m}^{-1}$ . 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  $\mu\text{m}$ ). Forces were  
920 calculated from the determined bead positions according to the equation,  $F = \kappa \cdot (x - x_o)$ ,  
921 where  $\kappa$  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  $\text{CO}_2$  controllable stage-top incubator (STXG-WELSX, Tokai Hit) was attached,  
925 allowing cells to be maintained at  $37^\circ\text{C}$  in a humidified, 5%  $\text{CO}_2$  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  $\text{cm}^{-2}$ . 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 ProLong<sup>TM</sup> Live  
931 Antifade Reagent (Invitrogen) at a 1:75 dilution, and  $2 \text{ mg mL}^{-1}$   $\beta$ -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 Mask<sup>TM</sup> 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  $\tau$ -SPAD single-photon avalanche diodes (PicoQuant). The  $\tau$ -SPADs were  
943 controlled by the SymPhoTime 64 software (PicoQuant). Images encompassing the nanotube and  
944 some of the cell body (typically  $1024 \times 512$  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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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  
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1267 wrote the original draft with inputs and revisions from JMH and PB.

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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  $\mu\text{m}$ . (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  $\mu\text{m}$ . (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  $\mu\text{m}$ .  
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 PIP<sub>2</sub>-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% PIP<sub>2</sub> (PIP<sub>2</sub>-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 PIP<sub>2</sub>-GUVs and on PS-GUVs. Each data point  
1324 represents one cluster. PIP<sub>2</sub>-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% PIP<sub>2</sub>), 2% PIP<sub>2</sub>-like, and 5% PIP<sub>2</sub>-like membranes. Membrane CG  
1328 beads in grey, PIP<sub>2</sub>-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 PIP<sub>2</sub> clusters (blue). (E) Probability of I-BAR domain aggregate size  
1331 to be  $< 5$  or  $\geq 5$  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% PIP<sub>2</sub>. 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  $\mu\text{m}$ .  
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  $\mu$ M, 10% - 27% AX488  
1344 labelled), fascin (250 nM), CP (25 nM) and profilin (0.6  $\mu$ M). GUV composition: TBX, 0.5%  
1345 TR-ceramide and 5% PIP<sub>2</sub>. Scale bars, 5  $\mu$ 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% PIP<sub>2</sub>. “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  $\mu$ m; (B, C) 2  $\mu$ 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 Mask<sup>TM</sup> 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 ( $S_{\text{avg}}$ ). (D) Measured  $S_{\text{avg}}$  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,  $S_{\text{avg}}$ , 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 ( $\Delta F$ ). 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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