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1 **Regulation of Kinesin-1 activity by *Salmonella enterica***
2 **effectors PipB2 and SifA**

3

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21

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24 membrane tubules

25 **Summary statement**

26 The Kinesin-1 motor protein plays a key role in controlling the membrane
27 exchanges necessary for the intracellular survival of *Salmonella*, a pathogenic
28 bacterium. This article highlights the complexity of Kinesin-1 regulation by
29 *Salmonella* effector proteins.

30 **Abstract**

31 *Salmonella enterica* is an intracellular bacterial pathogen. The formation
32 of its replication niche, composed of a vacuole associated with a network of
33 membrane tubules, depends on the secretion of a set of bacterial effector
34 proteins whose activities deeply modify the functions of the eukaryotic host
35 cell. By recruiting and regulating the activity of the Kinesin-1 molecular motor,
36 *Salmonella* effectors PipB2 and SifA play an essential role in the formation of
37 the bacterial compartments. In particular, they allow the formation of tubules
38 from the vacuole, their extension along the microtubule cytoskeleton and thus
39 promote membrane exchanges and nutrient supply. We have developed *in*
40 *vitro* and *in cellulo* assays to better understand the specific role played by
41 these two effectors in the recruitment and regulation of Kinesin-1. Our results
42 reveal a specific interaction between the two effectors and indicate that
43 contrary to what studies on infected cells suggested, interaction with PipB2 is
44 sufficient to relieve the autoinhibition of Kinesin-1. Finally, they **suggest the**
45 **involvement of other *Salmonella* effectors in the control of the activity of this**
46 **molecular motor.**

47

48 **Introduction**

49 The species *Salmonella enterica* is composed of a large number of
50 serotypes, including typhoid strains, which cause severe systemic disease
51 (typhoid fever) in humans, and non-typhoid strains responsible for acute
52 gastroenteritis. The latter may also cause systemic infection in hosts already
53 weakened by another viral or parasitic infection (Gordon et al., 2008). The
54 non-typhoid strain *S. Typhimurium* (*Salmonella enterica* subsp. *enterica*
55 serotype Typhimurium) is commonly used as a model for studying host-
56 pathogen interactions (LaRock et al., 2015).

57 *Salmonella* is an enteropathogenic bacterium. During the infectious
58 process, bacteria that have reached the gut cross the intestinal barrier,
59 preferably through the M cells of Peyer's patches (Lelouard et al., 2012), and
60 to a lesser extent by infecting enterocytes. They then migrate and are found in
61 a multitude of cell types, in particular cells of the immune system (Aussel et
62 al., 2011) in Peyer's patches, liver, spleen and also in epithelial cells, for
63 example in the gallbladder (Knodler et al., 2010).

64 The pathogenicity of *Salmonella* depends on its ability to replicate
65 intracellularly in a membrane-bound compartment called *Salmonella*-
66 containing vacuole (SCV). The membrane of the SCV has the particularity of
67 being enriched in lysosomal proteins. Infected cells show a rarefaction of
68 lysosomal vesicles which seem to have merged with the SCVs. This
69 recruitment of lysosomal membranes takes place thanks to the formation of
70 membrane tubules that are formed from the SCVs and extend on the network
71 of microtubules in the cells. These tubular membrane structures, called

72 *Salmonella*-induced tubules, promote exchange with lysosomal vesicles,
73 membrane recruitment and transport of material to the SCVs (Liss et al.,
74 2017) and ultimately provide the nutrients necessary for intracellular bacterial
75 replication (Noster et al., 2019).

76 This unique process is orchestrated by a series of bacterial proteins that
77 are injected into the host cell cytosol by a type III secretion system (T3SS),
78 encoded by the second pathogenicity island (T3SS-2). The T3SS-2 effector
79 proteins influence a large number of cellular processes such as gene
80 expression (Mazurkiewicz et al., 2008), localization of membrane proteins
81 (Bayer-Santos et al., 2016) or dynamics of the cytoskeleton (Abrahams et al.,
82 2006; Méresse et al., 2001). By interacting with the microtubule motor protein
83 Kinesin-1, the two effector proteins PipB2 (Henry et al., 2006) and SifA
84 (Boucrot et al., 2005) play a particularly important role in the formation and
85 function of *Salmonella*-induced tubules. Kinesin-1 is a hetero-tetrameric
86 ATPase composed of two heavy chains (KHC) and two light chains (KLC) that
87 promotes the transport of cargoes toward the growing end of microtubules.
88 PipB2 interacts directly with KLCs (Henry et al., 2006), while SifA binds to
89 SKIP (also known as PLEKHM2), an adaptor host protein that interacts with
90 KLCs (Boucrot et al., 2005; Dumont et al., 2010).

91 In the absence of cargo binding, Kinesin-1 adopts a folded, compact
92 conformation that is auto-inhibited and cytosolic (Cai et al., 2007). Cargo
93 binding relieves autoinhibition and results in an extended structure, releasing
94 KHC motor domains that engage with the microtubules (Hirokawa and Noda,
95 2008) and convert the chemical energy of ATP hydrolysis into motion along
96 microtubules. The mechanism of activation of Kinesin-1 is not yet fully

97 understood. It involves interactions between the cargo and both KHCs and
98 KLCs, the latter being involved in the inhibition of the enzymatic activity of the
99 KHCs. In the cellular context, binding of the cargo protein JPI1 to KLC is not
100 sufficient to activate Kinesin-1 and requires additional interactions of KHC with
101 FEZ1 (Blasius et al., 2007). The TPR domain of KLC recognizes a peptide
102 sequence composed of a tryptophan (Dodding et al., 2011) or a tyrosine
103 (Pernigo et al., 2018) residue flanked by acid residues, sequences that have
104 recently been named W-acidic and Y-acidic. The W-acidic motif originally
105 characterized in vaccinia virus protein A36 is also found in many adaptor
106 proteins such as SKIP, which contains two of them (Pernigo et al., 2013). A
107 recent study showed that SKIP can also interact with KHC when already
108 interacting with KLC and that this double interaction relieves the auto-
109 inhibition of Kinesin-1 (Sanger et al., 2017).

110 In *Salmonella*-infected cells, SifA and PipB2 are important for the
111 formation and/or elongation of *Salmonella*-induced tubules. Cells infected with
112 a mutant $\Delta pipB2$ are characterized by the formation of short tubules (Knodler
113 and Steele-Mortimer, 2005) while these are lacking or very rare in the
114 absence of SifA (Stein et al., 1996) or SKIP (Boucrot et al., 2005). The
115 specific impact of PipB2 and SifA on Kinesin-1 activation is still not
116 understood. However, based on previous observations and in particular
117 because in the absence of SifA, PipB2-bound Kinesin-1 accumulates on
118 SCVs, we proposed that the SifA/SKIP complex is necessary for the activation
119 of the Kinesin-1 recruited by PipB2 (Dumont et al., 2010; Leone and Méresse,
120 2011). In this study, we used *in vitro* and *in cellulo* approaches to investigate
121 the activation status of Kinesin-1 when recruited by PipB2 and SifA effectors.

122

123 **Results**

124 **Kinesin-1 is important for membrane dynamics and intracellular position** 125 **of SCVs.**

126 To better understand the role played by Kinesin-1 in the formation of
127 vesicles and tubules emerging from SCVs, we analysed the consequences of
128 the absence of the molecular motor in *Salmonella*-infected cells. For this
129 purpose, we differentiated macrophages from Hoxb8-immortalized
130 macrophage-committed progenitor cells (Redecke et al., 2013; Wang et al.,
131 2006). These cells were derived from C57BL/6 mouse expressing KIF5B
132 (Kinesin-1 heavy chain) in hematopoietic cells or not (Munoz et al., 2016). By
133 Western blotting, anti-KHC antibody recognized in KIF5B^{+/+} macrophages and
134 mouse brain cytosol a band between 100 and 150 kDa which was absent in
135 KIF5B^{-/-} macrophages (Fig. 1A), thus confirming the absence of functional
136 Kinesin-1.

137 The macrophages were infected with a *S. Typhimurium* strain (wild-type)
138 expressing GFP and a HA-tagged version of the T3SS-2 effector protein SseJ
139 and observed by confocal microscopy 14 hours after infection. GFP was used
140 to detect the presence of bacteria in cells. LAMP1 and SseJ were used as
141 membrane markers of bacterial compartments. While *Salmonella*-induced
142 tubules form in most infected epithelial cells, these membrane structures are
143 only rarely seen in non-activated macrophages (Knodler et al., 2003; Krieger
144 et al., 2014). In macrophages, effectors tend to be located on discrete
145 vesicles throughout the cell (Freeman et al., 2003; Ohlson et al., 2005).
146 Unsurprisingly, in KIF5B^{+/+} macrophages infected with wild-type *Salmonella*,

147 SseJ was present on SCVs and also at distance from SCVs in association
148 with vesicular structures (Fig. 1B). In contrast, SseJ was essentially localized
149 on SCVs in the absence of Kinesin-1 and also in KIF5B^{+/+} cells infected with
150 the Δ *sifA* mutant (Fig. 1B and 1C) as expected (Zhao et al., 2015). These
151 results indicate that Kinesin-1 is required for the mechanisms by which SseJ
152 traffics from SCVs to discrete vesicular structures. They also support the
153 previously proposed hypothesis (Dumont et al., 2010) that associates the
154 absence of vesicles/tubules in cells infected by a Δ *sifA* mutant with a low level
155 of activation of Kinesin-1 that, recruited by PipB2, accumulates on SCVs
156 (Henry et al., 2006).

157 We also observed an influence of Kinesin-1 on the position of SCVs.
158 While in the KIF5B^{+/+} and KIF5B^{-/-} macrophages, the wild-type SCVs were
159 located in the **juxtannuclear** region (Fig. 1C), they were grouped very
160 compactly and close to the nucleus in the absence of Kinesin-1 (Fig. 1B). This
161 indicates that Kinesin-1 has an anterograde transport activity that maintains
162 the SCVs in the **juxtannuclear** region but at some distance from the nucleus. In
163 KIF5B^{+/+} macrophages, two populations of Δ *sifA* SCVs were distinguished
164 according to whether or not they were positive for SseJ. The positive ones,
165 which are also those that accumulate Kinesin-1 (Zhao et al., 2015), showed a
166 localization much further from the nucleus than other vacuoles. This
167 difference was not found in the KIF5B^{-/-} macrophages where vacuoles
168 indistinctly adopt a **juxtannuclear** position (Fig. 1B, 1C). This shows that the
169 Kinesin-1 present on Δ *sifA* SCVs, although not very active, is nevertheless
170 responsible for the anterograde movement of bacterial vacuoles. Overall,
171 these results underline the importance of Kinesin-1 in the formation of

172 effector-positive membrane vesicles derived from SCVs, indicate that this
173 molecular motor is also necessary for the anterograde movement of SCVs
174 and confirm the low state of activation of PipB2-bound Kinesin-1 present on
175 Δ sifA SCVs.

176 **PipB2-bound Kinesin-1 is active**

177 PipB2 plays a key role in the recruitment of Kinesin-1 on SCVs (Boucrot
178 et al., 2005; Henry et al., 2006). However, it is still unknown whether Kinesin-1
179 is the only molecular motor recruited by PipB2 and whether this effector can
180 distinguish between the activation states of Kinesin-1 or play a role in the
181 activation of this molecular motor. To gain insights on the motor activity of
182 PipB2-bound proteins, we reconstituted an *in vitro* microtubule gliding assay.
183 It consists of a streptavidin-coated glass chamber successively filled with
184 biotinylated Δ 17PipB2 (Bio-PipB2), a fraction of molecular motors, fluorescent
185 microtubules and a motility buffer containing 1mM ATP (Fig. S1A). As a
186 positive control, the glass chamber was coated with the biotinylated motor
187 domain of *Drosophila* Kinesin heavy chain (K401-Bio). This constitutively
188 active recombinant protein {Berliner:1995iy, (Roux et al., 2002; Subramanian
189 and Gelles, 2007) induced a microtubule gliding with a velocity of 0.05 ± 0.01
190 $\mu\text{m/s}$ and a narrow distribution of gliding velocities (Fig. 2, S2A and Movie 1).
191 In the presence of Bio-PipB2 and MAP-depleted mouse brain cytosol as a
192 source of molecular motors, we observed persistent attachment and
193 sustained movement of the microtubules after injection of motility buffer (Fig.
194 2, S2B, S2C). The microtubules displayed a gliding motility throughout the
195 recorded period of 15 minutes (Movie 2) with a velocity of $0.22 \pm 0.01 \mu\text{m/s}$

196 (Fig. S2A), which is higher than that observed with the motor domain of
197 *Drosophila* KHC but inferior to the gliding speed of 0.5 - 0.7 $\mu\text{m/s}$ reported in
198 the literature in a similar assay using bovine Kinesin (Grummt et al., 1998).
199 When biotinylated BSA (Bio-BSA), our negative control, was injected instead
200 of PipB2, we noticed the gliding of a small fraction of microtubules (Fig. 2,
201 S2C) and the rapid decrease in the number of attached microtubules (Fig.
202 S2B, Movie 3). We concluded that the attachment and the movement of the
203 microtubules depend on PipB2.

204 To determine if Kinesin-1 is the cytosolic factor mediating microtubules
205 gliding, we fractionated a cytosol preparation enriched in microtubule-based
206 motors by centrifugation on a sucrose gradient. Fraction #7, which presented
207 the highest concentration of Kinesin-1 (Fig. S3A) was tested in the gliding
208 assay. We observed a velocity of $0.15 \pm 0.01 \mu\text{m/s}$, of the same order of
209 magnitude as that obtained with MAP-depleted mouse brain cytosol (Fig. 2,
210 S2A and Movie 4). To confirm the involvement of Kinesin-1 in the PipB2-
211 mediated motility, we prepared cytosol fractions from Hoxb8 macrophages.
212 KIF5B^{+/+} cytosol promoted the gliding of microtubules with a speed of $0.23 \pm$
213 $0.02 \mu\text{m/s}$ (Fig. 2, S2A and Movie 5). In the presence of KIF5B^{-/-} cytosol, a
214 small fraction of microtubules that partially attached sometimes showed random
215 mobility over a short period before detaching (Fig. 2, S2C, S2D and Movie 6).
216 Overall, these results indicate that Kinesin-1 is responsible for the gliding of
217 microtubules observed in this assay and that PipB2-bound Kinesin-1 is
218 capable of engaging microtubules and inducing their movement in the
219 presence of ATP.

220 **PipB2-bound Kinesin-1 is sufficient to pull tubules from giant**
221 **unilamellar vesicles**

222 The formation of *Salmonella*-induced tubules from SCVs requires an
223 intact microtubule network where tubules exhibit a bidirectional movement
224 (Drecktrah et al., 2008; Krieger et al., 2014; Rajashekar et al., 2008). Using a
225 minimal biomimetic membrane system *in vitro*, we tested whether the
226 PipB2/Kinesin-1 complex could provide enough force to generate membrane
227 tubule networks from giant unilamellar vesicles (GUVs). GUVs were made
228 fluorescent by addition of 1% β -BODIPY-DOPC and contained 0.1%
229 biotinylated-DOPE, making them capable of binding biotinylated proteins in
230 the presence of streptavidin (Fig. 3A). We modified the tube pulling assay
231 previously described (Leduc et al., 2004). Briefly, in a flow chamber covered
232 by fluorescent microtubules, we sequentially injected the biotinylated protein
233 in the presence of streptavidin, a motility buffer containing 1mM ATP, and
234 GUVs (Fig. S1B). We used **K401-Bio** and Bio-BSA as positive and negative
235 controls, respectively. In the presence of **K401-Bio**, we observed the
236 formation of an extended network of tubules, pulled from the GUVs, which
237 extended along the microtubules network (Fig. S4), while no tubule was
238 observed with Bio-BSA (Fig. 3B). These results validated the experimental
239 system. When using Bio-PipB2 pre-incubated with a Kinesin-1-containing
240 fraction we obtained networks of tubules similar to those observed with **K401-**
241 **Bio** (Fig. 3B). Taken together, these results confirm that PipB2-bound Kinesin-
242 1 is capable of engaging microtubules and demonstrate that the force
243 produced by the complex is sufficient to induce membrane tubules.

244 **PipB2 increases the activation state of Kinesin-1**

245 The vast majority of cellular Kinesin-1 is cytosolic and inactive in a folded
246 state that blocks its interaction with microtubules (Cai et al., 2007). Yet, our
247 results indicate that at least a part of PipB2-bound Kinesin-1 is active. This
248 can be due to the presence of a significant fraction of active Kinesin-1 in the
249 mouse cytosol and the capacity of PipB2 to indifferently bind active Kinesin-1
250 or auto-inhibited Kinesin-1. Another possibility is the capacity of PipB2 to
251 relieve the autoinhibition of the microtubule motor. To discriminate between
252 these hypotheses, we developed a microtubule-binding protein spin-down
253 assay to evaluate the fraction of cytosolic Kinesin-1 capable of engaging
254 microtubules (activated Kinesin-1) in the presence or absence of PipB2.
255 Briefly, *in vitro* polymerized microtubules were incubated with the proteins of
256 interest and sedimented through a glycerol cushion by ultracentrifugation. As
257 a control, incubations were performed in the absence of polymerized
258 microtubules.

259 We first analysed by SDS-PAGE and Western blotting the distribution of
260 **K401-Bio** in the supernatant and pellet fractions. This recombinant protein is
261 constitutively active (Roux et al., 2002; Subramanian and Gelles, 2007). The
262 presence of microtubules significantly increased the proportion of the motor
263 domain in the pellet (~40% compared to ~10% in the absence of
264 microtubules) (Fig. 4A). This result indicates that **K401-Bio** is capable of
265 engaging microtubules but remains in the supernatant in the absence of
266 microtubules. Next, we analysed the consequences of introducing Bio-PipB2
267 on the activation of Kinesin-1 in a mouse brain cytosolic fraction. In the
268 absence of microtubules, Kinesin-1 was not detectable in the pellet, whether

269 or not PipB2 was present (Fig. 4B). In the presence of microtubules,
270 approximately 40% of total Kinesin-1 was found in the pellet. This fraction was
271 increased to 90% in the presence of PipB2 (Fig. 4B), that was also present in
272 greater quantity in the pellet in the presence of microtubules (Fig. 4C). We
273 performed a dose-response experiment and observed a correlation between
274 the amount of PipB2 added and Kinesin-1 present in the pellet (Fig. 4D).
275 These results indicate that PipB2 activates Kinesin-1 and increases the
276 fraction of molecular motor that engages microtubules.

277 **PipB2 and SifA interact directly**

278 Since both interact with Kinesin-1 and influence, as the above results
279 show for PipB2, or may influence (SifA, (Schroeder et al., 2011)) the activity of
280 Kinesin-1, we considered the possibility of an interaction between SifA and
281 PipB2. HeLa cells were transfected with plasmids for the expression of GFP
282 or GFP-tagged PipB2 and Myc-tagged variants of SifA. We used cell lysates
283 to immuno-precipitate GFP proteins and analysed the associated proteins by
284 SDS-PAGE and anti-Myc Western blotting. SifA co-immunoprecipitated with
285 GFP-PipB2 but not with GFP (Fig. 5A), indicating a specific interaction
286 between the two effectors. We tested the two SifA domains (Ohlson et al.,
287 2008) and found an interaction of PipB2 with the N-terminal part of SifA (Fig.
288 5A). Finally, we expressed and purified GST-PipB2 and [His]₆-SifA from *E. coli*
289 and performed a GST pull-down assay to assess whether or not the
290 interaction is direct. We used raw or GST-coated beads as controls for the
291 specificity and the TPR domain of mouse KLC2 ([His]₆-KLC) as a positive

292 control for the interaction with GST-PipB2. Results (Fig. 5B) indicate a specific
293 direct interaction between SifA and PipB2.

294 **SifA and PipB2 do not influence their respective activities**

295 We checked if SifA alone or in combination with PipB2 and in the
296 presence of cytosol (a source of Kinesin-1 and SKIP) could bind and influence
297 the activity state of the molecular motor. Since the gliding and tubulation
298 assays used a form of PipB2 deleted of its first 17 amino acid residues (Bio-
299 PipB2), we verified that this domain is not necessary for interaction with SifA
300 (Fig. 5A). Then, we tested the ability of SifA to interact with Kinesin-1 or to
301 influence the interaction between the molecular motor and PipB2. For this,
302 streptavidin beads were coupled with Bio-PipB2, SifA-Bio or an equimolar
303 mixture of both effectors and incubated in the presence of cytosol. Beads-
304 bound Kinesin-1 was analysed by Western blotting using an antibody directed
305 against KHC (Fig. S3B). Kinesin-1 was present on beads coated with PipB2
306 but not on those coated with SifA. Furthermore, the presence of these two
307 effectors did not influence the recruitment of Kinesin-1 in any way. In the
308 microtubule gliding assay, SifA-Bio induced the binding of a low number of
309 microtubules that quickly detached (Fig. S3C and Movie 7). When mixed in an
310 equimolar amount to PipB2, SifA did not modify the PipB2-induced
311 microtubule binding and gliding (Fig. S3C). In the microtubule-binding protein
312 spin-down assay, SifA-Bio did not increase the pelleted fraction of Kinesin-1
313 (Fig. 4B). The conclusion of these experiments is that, under the experimental
314 conditions used, SifA is not capable of changing the activation state of
315 Kinesin-1 nor modulating the ability of PipB2 to activate Kinesin-1.

316 **PipB2 activates Kinesin-1 *in cellulo***

317 So far, our results indicate that PipB2 is able to increase the activity of
318 Kinesin-1 *in vitro*. We therefore checked whether the same was true in the
319 cellular context. For this, we transfected COS-7 cells with plasmids for the
320 expression of GFP-tagged *Salmonella* effectors and HA-tagged light (mouse
321 KLC2) and heavy (rat KIF5C) chains of Kinesin-1. After 24 hours we analysed
322 by fluorescence microscopy the impact of *Salmonella* effectors on the
323 activation of the molecular motor, i.e. its presence in the cytosol *versus* on
324 microtubules. Overexpressed KHC, KLC and Kinesin-1 (KHC/KLC) were
325 essentially cytosolic. KHC was also occasionally detected on microtubules
326 (Fig. S5). In agreement with previous studies (Henry et al., 2006; Knodler and
327 Steele-Mortimer, 2005) PipB2 was found in the cytosol and on vesicles on
328 which KLCs, which interact with PipB2, were detected unlike KHCs (Fig. 6A,
329 upper row). Compared to PipB (an homologue of PipB2 (Knodler et al.,
330 2003)), we observed in cells expressing PipB2 a dramatic change in the
331 localization of Kinesin-1, which then adopted a fibrous appearance and
332 colocalized with microtubules (Fig. 6A, middle row). The fraction of cells in
333 which we detected the presence of Kinesin-1 on microtubules increases from
334 2 % to about 65 % in the presence of PipB2, whereas no impact could be
335 observed in cells expressing PipB. In cells expressing KLC we also noted a
336 significant increase of the association with microtubules in the presence of
337 PipB2 (0 versus 20 %). This likely reflects the ability of exogenous KLC2 to
338 form functional hybrid molecular motor with endogenous KHC. Finally, we
339 also analysed the impact of SifA in combination with PipB2 or not. Like *in*
340 *vitro*, the expression of SifA had no impact on the localization of Kinesin-1 nor

341 on the ability of PipB2 to promote the engagement of Kinesin-1 on
342 microtubules (Fig. 6A, 6B, S5).

343 In order to validate these microscopic observations, we biochemically
344 analyzed the activation of Kinesin-1 in the cellular context as recently
345 described (Sanger et al., 2017). To do this, we lysed the transfected cells
346 under microtubule-stabilizing conditions, centrifuged the lysates and studied
347 the distribution of ectopically expressed proteins in the supernatant and
348 microtubule pellet. To validate the experimental system we expressed an
349 ATPase “rigor” mutant of KHC (KHC-R) that associates very stably with
350 microtubules (Guardia et al., 2016) and that was found almost exclusively
351 associated with microtubules in the pellet (Fig. 7). Compared to GFP or PipB,
352 our negative controls, we found that the expression of PipB2 significantly
353 increased the fraction of Kinesin-1 associated with microtubules, as was also
354 the case with SKIP, our positive control (Sanger et al., 2017) (Fig. 7). These
355 results confirm the microscopic observations and indicate that, like *in vitro*,
356 PipB2 promotes the activation of Kinesin-1 in the cellular context.

357

358 Discussion

359 Here we show that the interaction between the T3SS-2 effector PipB2
360 and the KLCs leads to the activation of Kinesin-1. This is demonstrated *in*
361 *vitro* by the activity of PipB2-recruited Kinesin-1 in microtubule gliding and
362 GUVs tubulation assays, the ability of PipB2 to increase the fraction of
363 Kinesin-1 bound to microtubules and the re-localization of Kinesin-1 from the
364 cytosol to the microtubule cytoskeleton in cells expressing PipB2.

365 Kinesin-1 mainly exists in a folded conformation that is auto-inhibited, i.e.
366 inactive for microtubule-based motility (Cai et al., 2007). Several examples
367 show that the interaction of KLCs with cargo participates in the activation of
368 the molecular motor (Blasius et al., 2007; Cai et al., 2007; Sanger et al.,
369 2017). From this point of view, the results obtained with PipB2 are not
370 surprising. However, they are not consistent with observations made in the
371 infectious context. Previous studies have shown that by recruiting Kinesin-1 to
372 the SCV, PipB2 induces elongation of tubules emerging from bacterial
373 vacuoles. However, in the absence of SifA, these tubules/vesicles do not form
374 and a PipB2-dependent accumulation of Kinesin-1 is observed on SCVs.
375 Based on these observations, we proposed (Boucrot et al., 2005) that: 1) the
376 Kinesin-1 present on $\Delta sifA$ SCVs is weakly or not active, which prevents the
377 formation of tubules/vesicles from SCVs; 2) the SifA/SKIP complex is
378 necessary for the activation of the Kinesin-1 recruited by PipB2. This model is
379 supported by a recent study (Sanger et al., 2017) demonstrating that multiple
380 interactions of the N-terminal part of SKIP with KLC and KHC promote
381 activation of Kinesin-1. The data presented here also support this model as

382 they show that the absence of functional Kinesin-1 in macrophages is
383 associated with the presence of SseJ on SCVs, a phenotype seen in the
384 absence of SifA. This strongly suggests that the Kinesin-1 recruited by PipB2
385 on $\Delta sifA$ SCVs is essentially inactive.

386 Our results thus show that the control of the activity of the Kinesin-1
387 present on SCV is more complex than initially thought and suggest the
388 involvement of other *Salmonella* effectors and/or host proteins. Among the
389 effectors (other than SifA and PipB2) known to influence vesicle/tubule
390 formation, two have activities compatible with possible involvement in the
391 regulation of Kinesin-1. Although not essential for the formation of *Salmonella*-
392 induced tubules, SseJ promotes the tubulation of the lysosomal compartment
393 when expressed ectopically with SifA (Ohlson et al., 2008). This event
394 depends on SKIP and microtubules, which suggests that SseJ exacerbates
395 the ability of SifA to induce tubulation (Boucrot et al., 2003) and reveals a
396 functional interaction that is important for the formation of tubules. SopD2,
397 when inactivated together with SifA, gives a strain ($\Delta sifA\Delta sopD2$) that
398 produces tubules. This phenotype is accompanied by a decrease in Kinesin-1
399 associated with SCVs and the appearance at the cellular periphery of vesicles
400 that are positive for PipB2 and Kinesin-1 (Schroeder et al., 2010). This
401 observation can be interpreted as a sign that SopD2 exerts an inhibitory
402 activity on Kinesin-1 linked to PipB2. It will be necessary to analyze the
403 impact of these effectors and possibly others also present on SCVs to
404 understand the regulation of Kinesin-1 activity in the context of infection.

405 Kinesin-1 is an abundant molecular motor, involved in a large number of
406 transport processes in different cell types and cell sites (for review see (Dinu

407 et al., 2007; Morfini et al., 2016; Verhey et al., 2001)). Regulatory
408 mechanisms for binding/detaching various cargoes are still poorly understood
409 and the protein patterns recognized by KLCs for binding cargoes have only
410 recently begun to be identified. A motif was originally found in the neural
411 protein calyculin-1 (Konecna et al., 2006). It consists of a peptide sequence
412 containing a tryptophan residue (W) flanked by negatively charged residues
413 (D or E). This W-acidic motif has been identified in pathogen or host proteins.
414 For example, it is duplicated in vaccinia virus protein A36 (Dodding et al.,
415 2011) which is necessary for recruitment of Kinesin-1 and transport of viral
416 particles to the periphery of infected cells (Rietdorf et al., 2001). The same
417 pattern is found in two copies in SKIP and is located in the N-terminal part of
418 the protein while the interaction domain with SifA is in the C-terminal half
419 (Boucrot et al., 2005; Pernigo et al., 2013). As no tryptophan residues are
420 used in the composition of the PipB2, the motif that allows this effector to
421 interact with KLC is necessarily different.

422 Several functional domains of PipB2 have been described. The N-
423 terminal part (residues 1 to 225, out of a total of 350) is sufficient for
424 translocation and localisation on SCVs and tubules (Knodler et al., 2003) but
425 not to cause the accumulation of LAMP1-positive vesicles at the cellular
426 periphery, a phenotype observed upon over-expression of PipB2. This
427 phenotype requires a pentapeptide, located in the last ten residues
428 (L₃₄₁FNEF₃₄₅) (Knodler and Steele-Mortimer, 2005). It should be noted that
429 this pentapeptide partially overlaps a sequence (E₃₄₄FYSE₃₄₈) that has the
430 characteristics of a Y-acidic motif. The latter has recently been identified in the
431 JIP1 protein and is, like the W-acidic motif, recognized by the KLC (Pernigo et

432 al., 2018). However, the involvement of this sequence in the interaction
433 between Kinesin-1 and PipB2 is uncertain since the deletion of the
434 pentapeptide, which partially covers this hypothesized Y-acidic motif, has only
435 a partial effect on the interaction between PipB2 and KLC (Henry et al., 2006).
436 All this information leads us to believe that, as already proposed (Knodler and
437 Steele-Mortimer, 2005), other determinants of PipB2 that have not yet been
438 identified are involved in the interaction with Kinesin-1 and the regulation of its
439 activity.

440 **Materials and methods**

441 **Antibodies and reagents.**

442 The antibodies and reagents used in this study were: mouse anti-Myc
443 (clone 9E10, homemade ascite), mouse anti-HA (clone 16B12, Covance),
444 mouse anti-KLC (clone L1, Chemicon International), mouse anti- β tubulin
445 (clone TUB 2.1, Sigma), mouse anti-histidine (clone HIS.H8; Thermo Fisher
446 Scientific), rat anti-HA (clone 3F10; Roche Molecular Biochemicals), goat anti-
447 mouse or anti-rabbit IgG coupled to peroxidase (Sigma), donkey anti-rat or
448 anti-mouse IgG conjugated to Alexa Fluor 546 or Alexa Fluo 647 (Jackson
449 ImmunoResearch), HRP-Conjugated Streptavidin (Thermo Fisher Scientific),
450 GFP-Trap[®] (Chomotek). The rabbit anti-KHC (KIF5B, PCP42) was generously
451 provided by R. Vale (University of California, San Francisco, CA). Unless
452 otherwise stated all reagents were purchased from Sigma-Aldrich (St. Louis,
453 MO).

454 **Cell lines and culture conditions.**

455 HeLa (ATCC CCL-2) and COS-7 cells (ECACC General Cell Collection,
456 # 87021302) were routinely grown in Dulbecco's modified Eagle's medium
457 (Gibco-BRL) supplemented with fetal calf serum 10% (Gibco-BRL) and
458 glutamine 2 mM.

459 Hoxb8-immortalized macrophage-committed progenitor cells were
460 derived from C57BL/6 mouse either wild-type or not expressing KIF5B in
461 hematopoietic cells following the procedure described by Redecke et al.
462 (Redecke et al., 2013). In brief, bone marrow cells from the femurs were pre-

463 stimulated for 48 h in RPMI 1640 based medium with 250 ng/mL SCF, 10
464 ng/mL IL3 and 20 ng/mL IL6. Ficoll-purified mononuclear cells were subjected
465 to spinoculation with 1 mL of ER-Hoxb8 retrovirus. Infected progenitors were
466 cultured in Progenitor Outgrowth Medium (RPMI 1640 with 10% FCS, 50 μ M
467 2-mercaptoethanol, 2 mM glutamine, 1 μ M β -estradiol and 2 % GM-CSF-
468 conditioned medium from B16 melanoma expressing the Csf2 cDNA).
469 Immortalized myeloid progenitors were selected by transferring nonadherent
470 progenitor cells every 3 days to a new well in a six-well culture plate over 3
471 weeks to produce immortalized macrophage progenitor lines. Differentiation to
472 macrophages was performed for 7 days in the same base medium free of β -
473 estradiol and GM-CSF but containing 10 % M-CSF-conditioned medium from
474 L929 cells.

475 **Bacterial strains, oligonucleotides and plasmids.**

476 A wild-type *S. enterica* serovar Typhimurium 12023 strain expressing
477 SseJ-HA from the chromosome was transformed with pFPV25.1 (Valdivia and
478 Falkow, 1996) for GFP expression. The Δ *sifA* mutant (#AAG003G) was
479 obtained by P22 transduction of Δ *sifA::kan*. The oligonucleotides and
480 plasmids used in this study are described in Tables S1 and S2, respectively

481 **Bacterial infection of Hoxb8 macrophages and immunofluorescence.**

482 Hoxb8-immortalized cells were seeded in 6-well plates with 12 mm
483 diameter glass coverslips and differentiated for 7 days. Cells were *Salmonella*
484 infected and processed for immunolabelling and fluorescence confocal
485 microscopy as described previously (Zhao et al., 2016).

486 **Cloning, expression and purification.**

487 The pDEST17-Bio (V276) vector was obtained by modifying the
488 pDEST17 Gateway destination vector by PCR to insert a biotin acceptor
489 peptide (15-mer GLNDIFEAQKIEWHE) between the [His]6 tag and the attR1
490 site. Δ 17PipB2 and SifA Δ 6Bio were amplified by PCR from *Salmonella* 12023
491 wild-type genomic DNA using the primer pairs O786 / O312 and O299 / O806,
492 respectively. The primer SifA Δ 6BioRev was designed to add the biotin
493 acceptor peptide sequence at the C-terminal. These PCR products were then
494 inserted in pDONRZeo resulting in the pDONR- Δ 17PipB2 (C1141) and
495 pDONR-SifA Δ 6-Bio (C1176). By recombination of pDONR- Δ 17PipB2 with
496 pDEST17-Bio and pDON-SifA Δ 6-Bio with pDEST17, the final plasmids
497 p[His]6-Bio- Δ 17PipB2 (C1143) and p[His]6-SifA Δ 6Bio (C1177) were obtained.
498 pWC2 (K401-Bio-H6) was a gift from Jeff Gelles (Addgene plasmid #15960)
499 (Subramanian and Gelles, 2007).

500 Rosetta™(DE3)pLysS competent cells (Novagen) containing pDEST17-
501 SifA Δ 6Bio or pWC2 (for the production of SifA-Bio and **K401-Bio**, respectively)
502 were grown overnight at 25°C in MagicMedia™ E. coli expression medium
503 (Thermo Fisher) supplemented with ampicillin 100 µg/mL and D-biotin 2.5
504 µg/mL. Rosetta containing pDEST17Bio- Δ 17PipB2 (for the production of Bio-
505 PipB2) was grown in 2x YT medium (Fisher Scientific) also supplemented with
506 ampicillin and D-biotin. At OD_{600nm}=0.8, overnight protein expression was
507 induced by adding 0.5 mM IPTG at 25°C. Proteins were purified with Hi-Trap
508 Chelating-Ni (GE Healthcare Life Sciences) on FPLC (ÄKTA Start, GE
509 Healthcare Life Sciences) using standard procedure. Finally, Bio-PipB2 was
510 dialyzed against 50 mM imidazole, pH 6.7, 50 mM KCl, 4 mM MgCl₂, 2 mM

511 EGTA, 10 mM β -mercaptoethanol and 20 % glycerol. **K401-Bio** was dialyzed
512 against the same buffer with 50 nM ATP. SifA-Bio was dialyzed as previously
513 described (Diacovich et al., 2009). Proteins were snap-frozen in liquid N₂ and
514 were stored at -80°C.

515 **Microtubule-Associated Protein (MAP)-depleted mouse brain cytosol** 516 **preparation.**

517 Mouse brains were washed several times with PBS, homogenized in 1
518 mL/mg of Lysis Buffer (50 mM Imidazole pH 6.7, 1 mM MgCl₂, 2 mM EGTA, 1
519 μ M DTT, 250 mM sucrose) and centrifuged at 10,000 x g, 4°C, for 20 minutes.
520 Proteinase inhibitors (Thermo Scientific) and 10 μ M paclitaxel were added to
521 the supernatant that was incubated for 30 minutes at 37°C. Finally, it was
522 centrifuged at 107,000 x g for 30 minutes at 25°C (Optima MAX-XP; Beckman
523 Coulter, Inc.) and the supernatant was kept at -80°C.

524 **Fractionation of microtubule-based motor proteins**

525 Mouse brains were homogenized in 1 mL/g PME (100 mM Pipes pH 6.9,
526 2 mM EGTA, 1 mM MgSO₄, 1 mM DTT, 1 mM GTP). The homogenate was
527 centrifuged at 100,000 x g for 30 minutes at 4°C and the supernatant
528 incubated for 30 minutes at 37°C with 2 units/mL apyrase and 20 μ M
529 paclitaxel and 30 extra minutes with 0.5 mM adenylyl-imidodiphosphate
530 (AMP-PNP). After centrifugation at 39,000 x g for 30 minutes at 30°C, the
531 pellet was resuspended in PME containing 0.1 mM AMP-PNP and 20 μ M
532 paclitaxel and centrifuged again. The pellet was resuspended in a small
533 volume of PME with 20 μ M paclitaxel and 10 mM Mg-ATP, incubated for 30
534 minutes at 37°C and centrifuged as in the previous steps. The supernatant

535 was loaded on a linear sucrose gradient (5 - 20 % in PME with 1mM Mg-
536 ATP), centrifuged at 31,500 rpm in a Beckman SW41 rotor for 16 hours at 4°C
537 and finally fractionated in aliquots of 0.5 mL that were stored at -80°C.

538 **Microtubules gliding assay.**

539 Polymerized microtubules were prepared by mixing rhodamine-tubulin
540 (Cytoskeleton Inc.) with purified porcine tubulin (Cytoskeleton Inc.) at a ratio
541 1:100 in BRB80 buffer (80 mM Pipes pH 6.7, 1 mM MgCl₂, 1 mM EGTA) with
542 10 % glycerol and 1 mM GTP and incubation at 37 °C for 20 minutes, followed
543 by addition of 10 μM paclitaxel and incubation for 20 extra minutes.
544 Microtubules were kept a room temperature protected from light until use. The
545 gliding assay was performed in a 10 μL flow chamber built with a glass slide,
546 coverslip and parafilm spacers in between, joined by heating at 150°C for a
547 few seconds. It was pre-coated with streptavidin 1 mg/mL for 5 minutes and
548 washed 3 times with wash buffer (BRB80 buffer pH 6.7, 0.15 mg/mL casein, 5
549 mM DTT, 10 μM paclitaxel). 10 μL of Protein buffer (20 mM Tris-HCl pH 7.6,
550 300 mM NaCl, 10 mM β-mercaptoethanol, 2 mM EGTA, 20 % glycerol)
551 containing 20 μM K401-Bio or 5 μM Bio-PipB2 or 5 μM SifA-Bio were injected,
552 incubated for 5 minutes and washed 3 times with the wash buffer. 10 μL of a
553 Kinesin-containing fraction was injected in the chambers containing Bio-PipB2
554 or SifA-Bio, incubated for 5 minutes and washed 3 times with the wash buffer.
555 10 μL rhodamine-microtubules previously diluted 1:100 (0.01 mg/mL) in wash
556 buffer were injected, incubated for 5 minutes and washed 3 times. Right
557 before recording the microtubules movement, motility buffer (BRB80 buffer pH
558 6.7, 0.15 mg/mL casein, 5 mM DTT, 10 μM paclitaxel, 0.18 mg/mL catalase,

559 0.37 mg/mL glucose oxidase, 10 mM glucose and 1 mM ATP) was introduced
560 into the flow-chamber and the chamber sealed. Microtubules were observed
561 using an epifluorescence microscope (Axio-observer; Zeiss) and time-lapses
562 (1 images / 30 seconds) were registered during periods between 2 to 30
563 minutes. Microtubule tracking was performed using FIJI (MTrackJ plugging,
564 10 microtubules per condition) and velocity of movement or quantification of
565 bound microtubules were determined.

566 **Protein binding and membrane tube pulling from Giant Unilamellar** 567 **Vesicles.**

568 Giant Unilamellar Vesicles (GUVs) were prepared by electroformation
569 (Angelova et al., 1992) with a lipid composition of 1,2-Dioleoyl-sn-Glycero-3-
570 Phosphocholine 1 mM (DOPC; Avanti Polar Lipids) supplemented with 1 % of
571 BodipyFL-C5-HPC (Fisher Scientific) and 0.1 % of the biotinylated lipid 1,2-
572 Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(Cap Biotinyl) (Biot-Cap-
573 DOPE; Avanti Polar Lipids). 10 μ L microtubules (1 mg/mL) in IMI buffer (50
574 mM Imidazole pH 6.7, 50 mM NaCl, 2 mM EGTA, 1 mM $MgCl_2$, 10 μ M
575 Paclitaxel) were injected in the chamber and incubated for 10 minutes before
576 injecting 10 μ L Casein (7 mg/mL in IMI buffer). 10 μ L of Protein buffer with
577 **K401-Bio** 20 μ M and streptavidin 7 μ M or 10 μ L of Kinesin-containing fraction
578 with Bio-PipB2 17 μ M or Bio-BSA 20 μ M and streptavidin 7 μ M were injected
579 and incubated for 15 minutes. Finally, 10 μ L of motility buffer (IMI buffer pH
580 6.7, 5 mM DTT, 10 μ M Paclitaxel, 1 mM ATP, 0.18 mg/mL catalase, 0.37
581 mg/mL glucose oxidase and 10 mM glucose) and GUVs 1:10 v/v were
582 sequentially injected. The chamber was sealed and kept at 45° from the

583 horizontal for few minutes to enable GUVs to settle before microscopic
584 observation.

585 **Microtubule-Binding Protein Spin-down Assay.**

586 10 μ L MAP-depleted mouse brain cytosol, 1 μ M Bio-PipB2 or 1 μ M SifA-
587 Bio, 10 μ M paclitaxel and 0.5 mM AMP-PNP in BRB80 buffer, pH 7.2 (final
588 volume of 50 μ L) were incubated for 30 minutes at room temperature in the
589 presence or not of 5 μ L microtubules (1 mg/mL). Bio-KHC was used as
590 positive control. Reaction mixes were then ultra-centrifuged through a glycerol
591 cushion (BRB80 buffer, 60 % glycerol and 10 μ M paclitaxel) for 40 minutes at
592 100,000 x g, 25 °C. Supernatant and pellet were kept, mixed/resuspended
593 with Laemmli sample buffer and 20 μ L of each sample were analyzed by
594 SDS-PAGE and Western blotting.

595 **Transfection of cells**

596 HeLa and COS-7 cells were transfected using FuGENE® 6 following
597 manufacturer's protocol.

598 **Coimmunoprecipitation of proteins expressed in HeLa cells.**

599 Transfected HeLa cells were washed with PBS, scraped with a rubber
600 policeman, centrifuged for 5 minutes at 400 g and resuspended in 400 μ L
601 lysis buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-
602 40) supplemented with a protease inhibitor cocktail. After 30 min at 4 °C cell
603 lysates were centrifuged at 20.000 x g for 10' at 4°C. 10 % of the lysates were
604 boiled in Laemmli sample buffer. Immunoprecipitations were performed with
605 GFP-Trap® beads that contain a recombinant alpaca anti-GFP antibody

606 covalently coupled to agarose beads following manufacturer's protocol.
607 Immunoprecipitated proteins were analysed by SDS-PAGE and Western
608 blotting using an appropriate antibody.

609 **Streptavidin Beads Pull-Down**

610 Bio-PipB2 or SifA-Bio or a combination of both proteins in a ratio 1:1
611 were incubated with streptavidin-agarose beads for 30 minutes at room
612 temperature. As negative controls, the same protocol without any protein was
613 performed. The beads were pelleted at 2,000 x g for 2 minutes, incubated with
614 10 μ M D-biotin and washed three times with BRB80 buffer. Then they were
615 incubated with MAP-depleted mouse brain cytosol for 30 minutes at room
616 temperature and wash 3 times as done before. Finally, beads were
617 resuspended in Laemmli sample buffer and Western blotting was performed
618 to analyse the bound proteins.

619 **Statistical Analyses**

620 Statistical analyses were performed with Prism 6 software (GraphPad).

621 **Acknowledgments**

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623 providing plasmids for expression HA-tagged light (mouse KLC2) and heavy
624 (rat KIF5C) chains of Kinesin-1, Hans Häcker (St. Jude Children's Research
625 Hospital, Memphis, Tennessee, USA) for the plasmid MSCV-ERHBD-Hoxb8,
626 Gae l M nasch  (Imagine Institute, Paris) for providing bone marrow
627 extracted from C57BL/6 mice not expressing KIF5B in hematopoietic cells,

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634 **Competing interests**

635 No competing interests declared.

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641 0023-01 to SM).

642 **Figure Legends**

643 **Figure 1. Impact of the absence of Kinesin-1 in *Salmonella*-infected**
644 **macrophages.**

645 (A) Kinesin heavy chain is not detected in KIF5B^{-/-} Hoxb8 macrophages.
646 Western blotting showing KHC expression in cell lysates of wild-type
647 (KIF5B^{+/+}) or KIF5B^{-/-} Hoxb8 macrophages and mouse brain cytosol. Actin
648 was used as loading control. (B and C) KIF5B^{+/+} and KIF5B^{-/-} Hoxb8
649 macrophages (Mφ) were infected with wild-type or Δ sifA strains of *S.*
650 Typhimurium expressing GFP from a plasmid and SseJ-2HA from the
651 chromosome. Cells were fixed at different times post-infection and
652 immunostained for LAMP1 and HA. (B) Cells (14 h post-infection) were
653 imaged by confocal microscopy for LAMP1 (red), SseJ (green), *Salmonella*
654 (white) and nuclei (blue). Magnified insets showing single labelling are
655 presented below each image. In the absence of functional Kinesin-1, wild-type
656 SCVs form a compact cluster very close to the nucleus while SseJ-positive
657 Δ sifA SCVs which, in KIF5B^{+/+} macrophages are at the periphery (arrows), are
658 located in the juxtannuclear area. Scale bar, 20 μm or 10 μm for the magnified
659 insets. (C) Percentages of infected cells with SseJ-positive SCVs (left graph)
660 or SseJ-positive SCVs located at the cell periphery (right graph). Phenotypes
661 were scored at three infection times. SCVs were counted as positive for SseJ
662 when the labelling for this effector identified the vacuolar compartment
663 enclosing the bacteria and considered peripheral when they were in the outer
664 third of the cytoplasmic space. Results are the means ± SD of three
665 independent experiments. A two-way ANOVA was used to compare for each

666 time of infection and bacterial strain the results obtained in cells expressing or
667 not KIF5B. *P* values: ns, *P*>0.05; *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.

668 **Figure 2. PipB2-bound Kinesin-1 is capable of gliding microtubules *in***
669 ***vitro***

670 As positive control, the chamber was coated with the motor domain of
671 Kinesin-1 (K401-Bio, upper row). Otherwise, glass chambers were coated
672 with BSA (Bio-BSA, negative control), or PipB2 (Bio-PipB2) and filled with
673 MAP-depleted mouse brain cytosol, fraction #7 enriched in microtubule-based
674 motor proteins or cytosol prepared from KIF5B^{+/+} or KIF5B^{-/-} Hoxb8
675 macrophages. Subsequently, rhodamine-microtubules and motility buffer
676 containing ATP were injected. Microtubule gliding was observed at 30-second
677 intervals for periods ranging from 2 to 30 minutes. The panel shows, for each
678 condition, the tracking of 4 microtubules over a 2-minute period and presents
679 images at 0, 60 and 120 seconds. To facilitate the visualization of the
680 movement, the minus end of these microtubules has been marked with a
681 coloured dot and its position at times 0, 30, 60, 90 and 120 sec indicated in
682 the last column. A rapid detachment of the microtubules was observed in the
683 absence of PipB2 (Bio-BSA) (orange tracks and yellow dots for the last
684 detected point). The mean speed ± SD of movement of the microtubules is
685 indicated in the last column. Scale bar, 20 μm.

686 Experimental repeats: K401-Bio, n > 9; MAP-depleted cytosol + Bio-
687 BSA, n= 3; MAP-depleted cytosol + Bio-PipB2, n > 15; Fraction#7 + Bio-
688 PipB2, n > 6; KIF5B^{+/+} macrophage cytosol + Bio-PipB2, n > 3; KIF5B^{-/-}
689 macrophage cytosol + Bio-PipB2, n > 3. Independent preparations: K401-Bio,

690 n = 2; MAP-depleted cytosol, n = 3; Bio-PipB2, n = 2; Fraction^{#7}, n = 2;
691 KIF5B^{+/+} macrophage cytosol, n = 1; KIF5B^{-/-} macrophage cytosol, n = 1.

692 **Figure 3. PipB2-bound Kinesin-1 is capable of pulling membranous**
693 **tubules from GUVs *in vitro***

694 (A) Representative confocal images of GUVs at the equatorial plane.
695 GUVs were made fluorescent by the presence of BodipyFL-C5-HPC (green)
696 in their composition and incubated or not in the presence of Bio-PipB2. The
697 presence of Bio-PipB2 on the surface of the GUV was revealed by the
698 addition of NTA-Atto 550 (red) that binds the [His]6 tag of the effector. Scale
699 bar, 10µm. (B) Membranous tubules are pulled from GUVs in the presence of
700 PipB2 and cytosol. GUVs were coated with K401-Bio or with Bio-BSA / Bio-
701 PipB2 in the presence of fraction ^{#7} enriched in microtubule-based motor
702 proteins and injected in the chamber coated with microtubules. K401-Bio and
703 Bio-BSA were used as positive and negative controls, respectively. Confocal
704 fluorescence images for the GUV membrane (green) and microtubules (red)
705 were taken in the microtubules plane. Long membrane tubules following
706 microtubule tracks were induced by K401-Bio and fraction ^{#7} in the presence
707 of Bio-PipB2 but not in the presence of Bio-BSA. Scale bar, 20 µm.
708 Experimental repeats: K401-Bio, n > 9; Fraction ^{#7} + Bio-BSA, n = 3; Fraction
709 ^{#7} + Bio-PipB2, n > 3. Independent preparations: K401-Bio, n = 2; Bio-PipB2,
710 n = 2; Fraction^{#7}, n = 2.

711 **Figure 4. PipB2 increases Kinesin-1 activation level**

712 A microtubule spin-down assay was used to assess changes in the
713 activation level of Kinesin-1. Microtubules were incubated with K401-Bio (A)

714 or with MAP-depleted mouse brain cytosol (**B - D**) supplemented or not with
715 Bio-PipB2 or SifA-Bio. Samples were ultra-centrifuged on glycerol cushions
716 and the supernatants (S) and pellets (P) were analysed by Western blotting
717 for the presence of biotinylated proteins (Strep) or KHC. (**D**) Increasing
718 amounts of PipB2 were added. Protein bands of Western blots were
719 quantified using Image J and the fraction of KHC present in pellets was
720 determined. The mean values \pm SEM of three independent experiments were
721 plotted.

722 **Figure 5. PipB2 interacts with SifA**

723 **(A)** PipB2 interacts with the N-terminal domain of SifA. HeLa cells were
724 transfected with plasmids for the ectopic expression of GFP or variants of
725 GFP-PipB2 and Myc-SifA. Immunoprecipitations were performed with GFP-
726 Trap[®] beads. Input (top panel) and bound proteins (middle panel) were
727 analyzed by Western blotting using anti-Myc. A Coomassie blue staining of
728 the PVDF membrane after Western blotting is presented (bottom panel). Note
729 that despite the removal of the N-terminal end, Δ 17PipB2 has a higher
730 apparent molecular weight than PipB2. This is due to cloning in the Gateway
731 system and the presence of a large linker between the GFP tag and PipB2
732 (see Table S2). **(B)** PipB2 and SifA interact directly. [His]₆-SifA or [His]₆-KLC
733 (positive control) were incubated with GST or GST-PipB2 immobilized on
734 glutathione beads. Purified and pulled-down proteins were analyzed by
735 Western blotting with an anti-[His]₆ antibody.

736 **Figure 6. PipB2 re-localizes Kinesin-1 onto microtubules**

737 COS-7 cells were transfected with plasmids for the ectopic expression of
738 HA-tagged KLC, KHC, or KLC and KHC (Kinesin-1) and of a GFP-tagged
739 effector (PipB2, SifA or PipB). **(A)** Fixed cells were immunostained and
740 imaged by confocal microscopy for GFP (green), HA (red), tubulin (blue). For
741 the cells of interest, a yellow line delimits the position of the nuclei (N).
742 Magnified insets showing single labelling are presented. Bar, 20 μm or 10 μm
743 for the magnified insets. **(B)** The presence of HA labelling on microtubules
744 was scored. Only transfected cells expressing both GFP- and HA- tagged
745 proteins were considered. Values are means \pm SD of at least three
746 independent experiments. For statistical analysis, paired t tests were used to
747 compare the means. *P*-values: ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

748 **Figure 7. PipB2 activates Kinesin-1 in the cellular context**

749 COS-7 cells were transfected with plasmids for the ectopic expression of
750 HA-tagged KLC and KHC and other proteins: GFP-PipB2, GFP and GFP-
751 PipB (negative controls) or GFP-SKIP(1-310) (positive control). An HA-tagged
752 ATPase “rigor” mutant of KHC (KHC-R) was used as positive control for the
753 experimental set-up. **(A)** Transfected cells were lysed under microtubule
754 stabilizing conditions, ultra-centrifuged on glycerol cushions and the
755 supernatants and pellets were analysed by Western blotting for the presence
756 of HA-tagged proteins (KLC, KHC, KHC-R), GFP-tagged proteins and tubulin.
757 **(B)** Protein bands of Western blots were quantified using Image J and the
758 fraction of KHC present in pellets was determined. The mean values \pm SD of
759 three independent experiments were plotted. A Dunnett’s test was used to

760 compare the fractions of activated Kinesin-1 with those of the negative control
761 (GFP). *P* values: ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$.

762 **References**

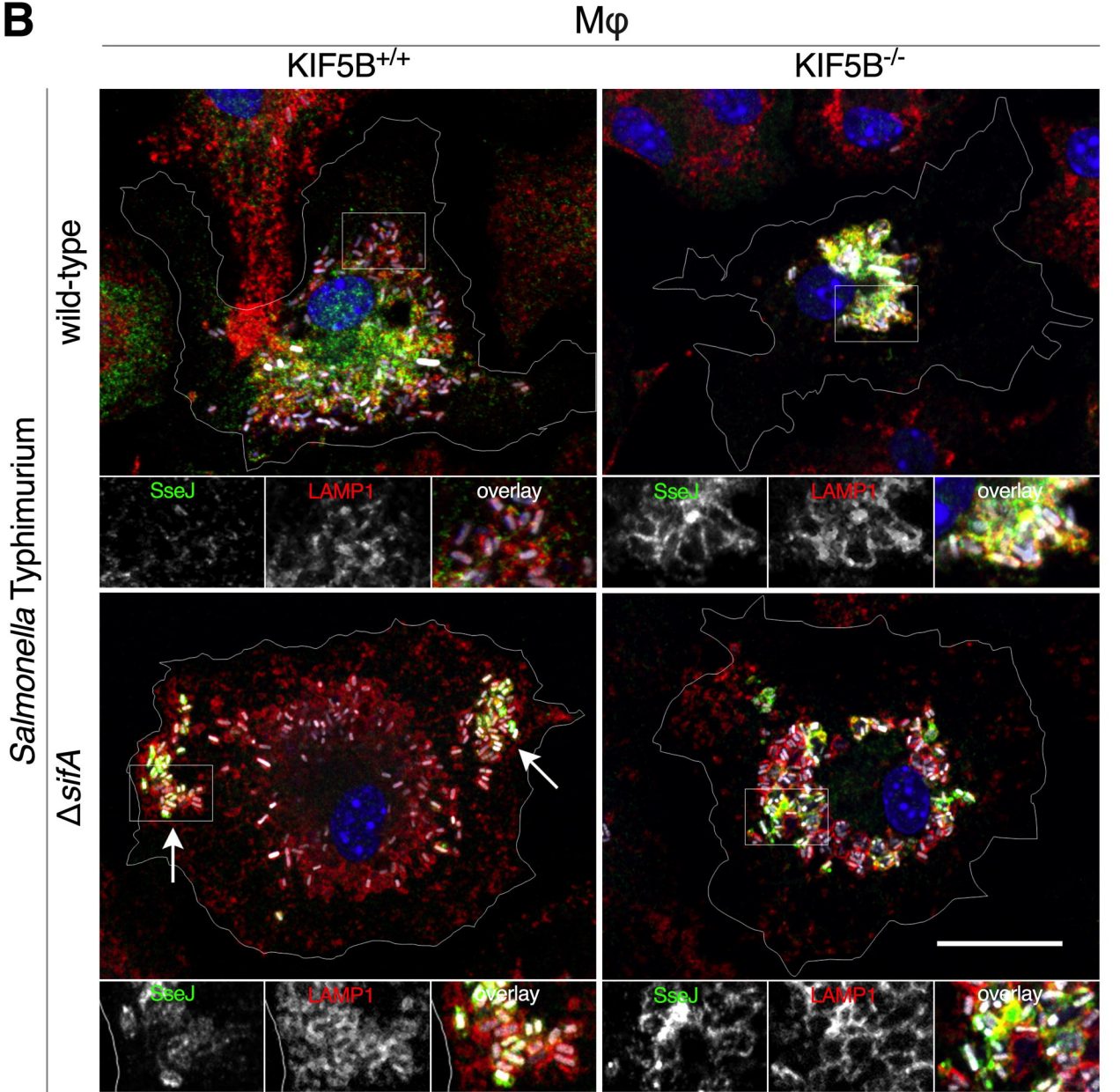
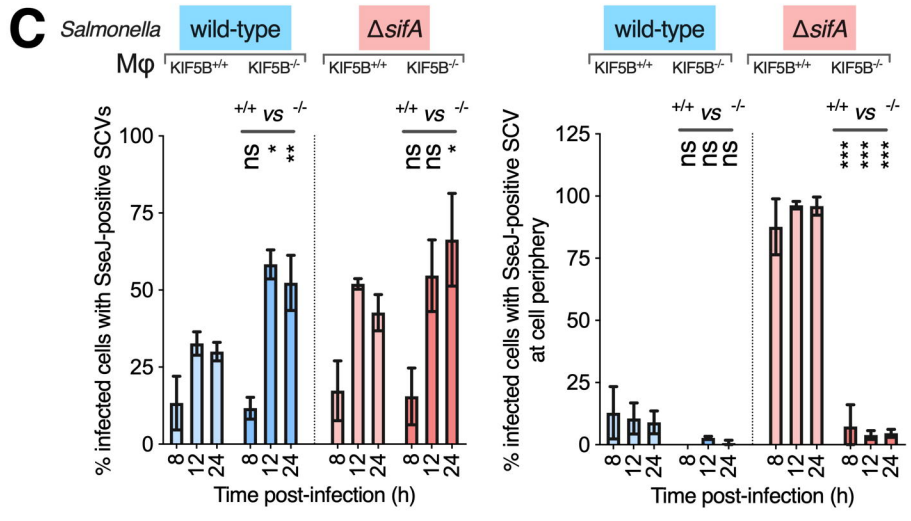
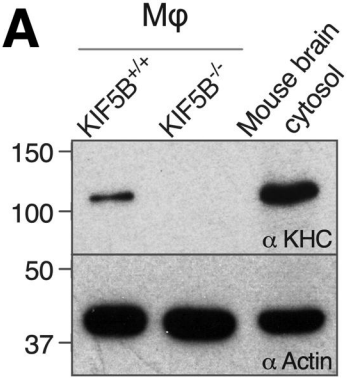
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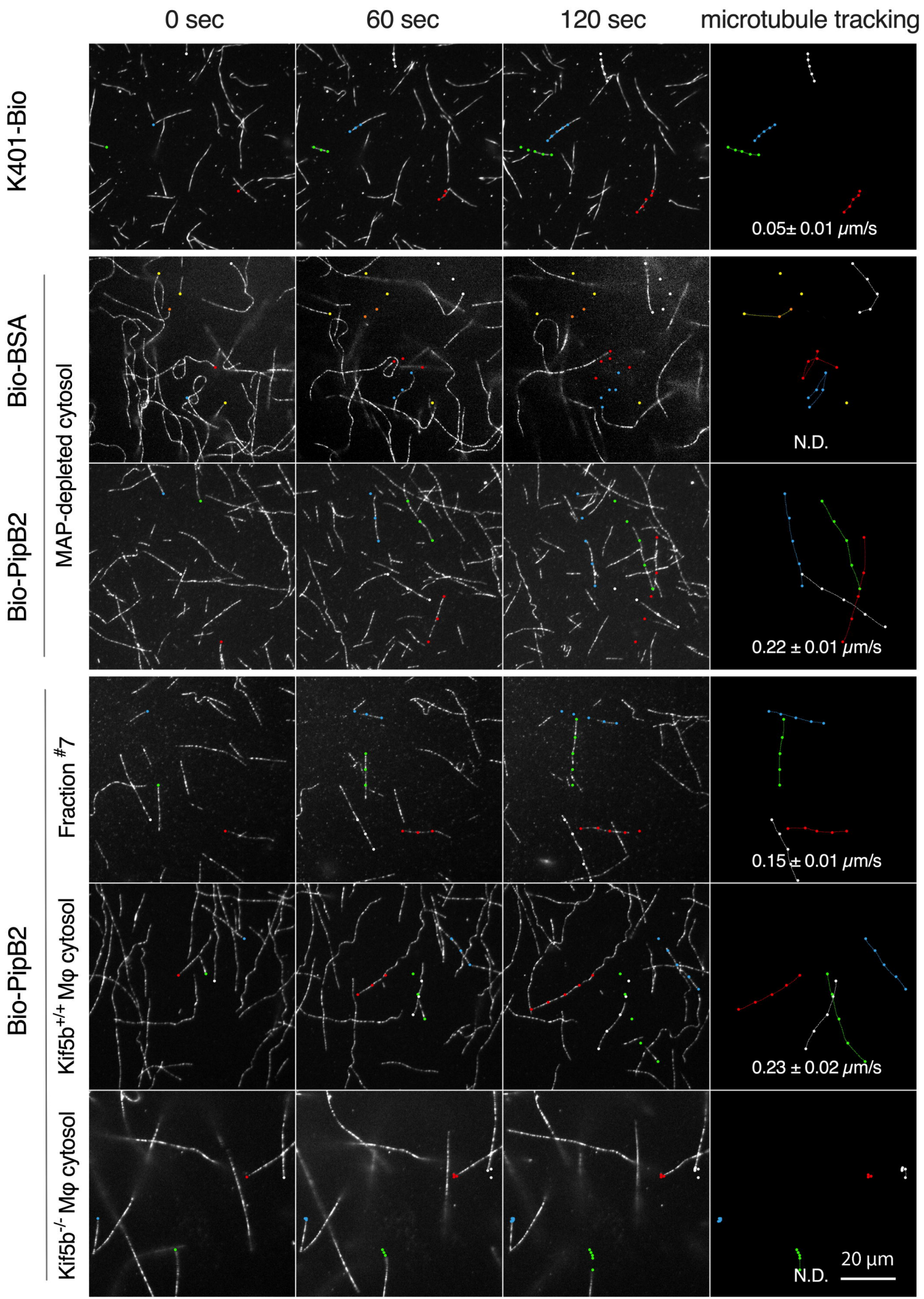
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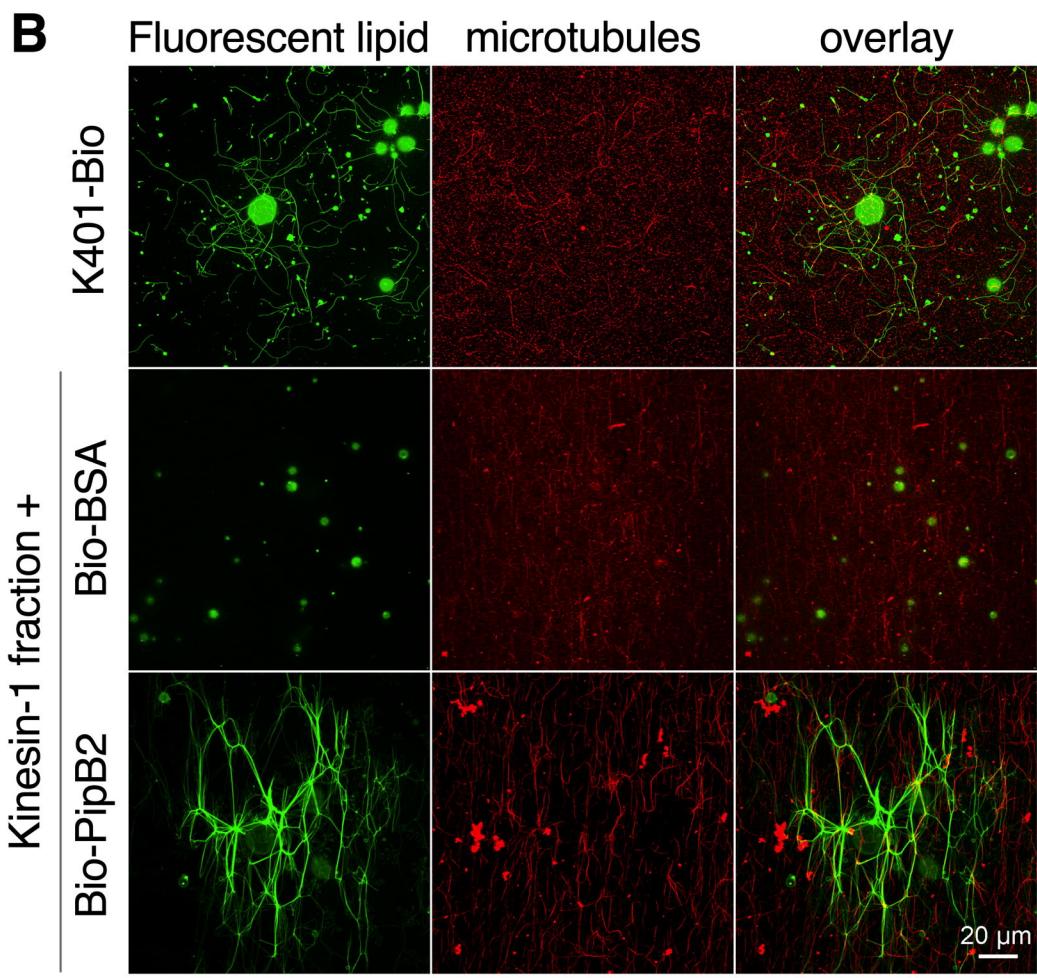
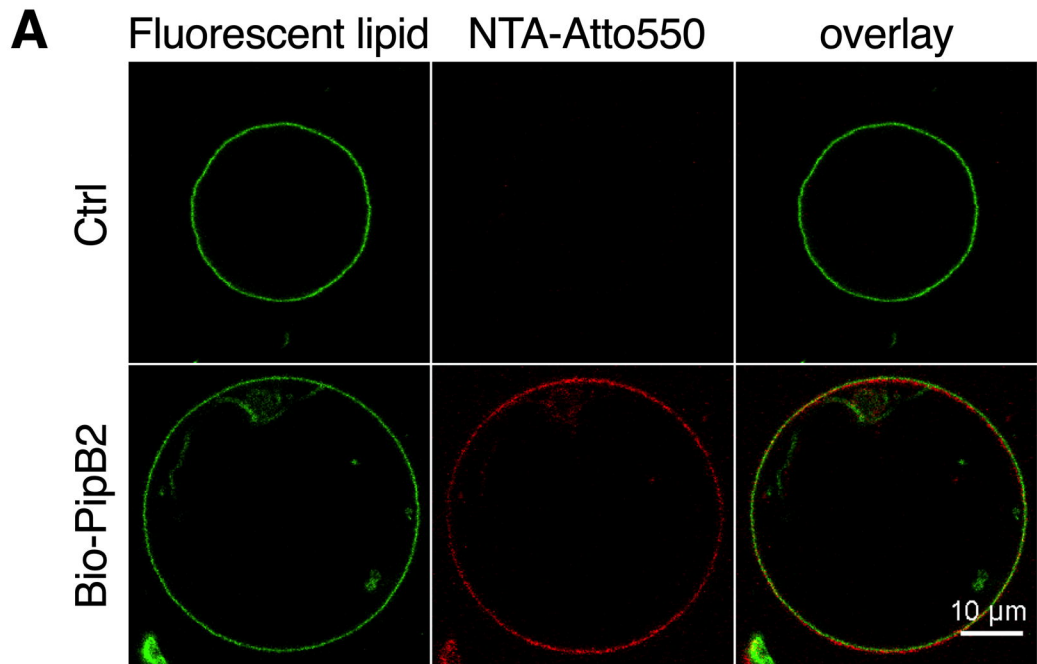
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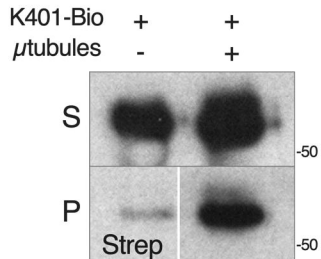
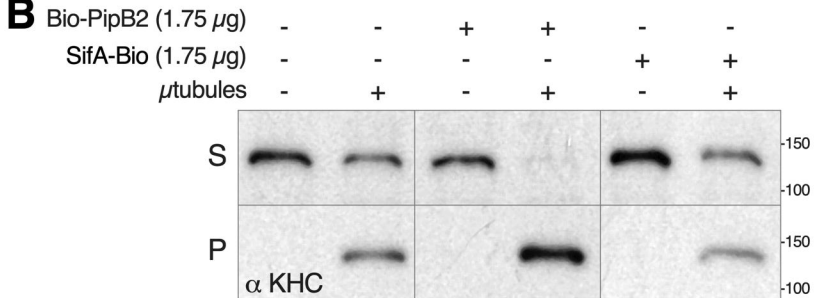
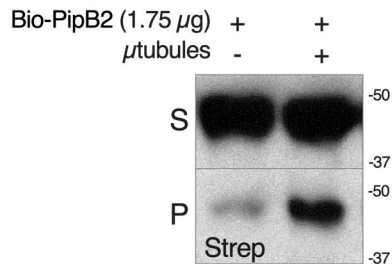
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