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Pryor, Paul Robert [orcid.org/0000-0001-9123-8329](https://orcid.org/0000-0001-9123-8329) (2017) The Salmonella effector SseJ disrupts microtubule dynamics when ectopically expressed in Normal Rat Kidney cells. PLoS ONE. ISSN 1932-6203

<https://doi.org/10.1371/journal.pone.0172588>

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1 The *Salmonella* effector SseJ disrupts microtubule dynamics when ectopically  
2 expressed in Normal Rat Kidney cells.

3

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16 Short title: SseJ disrupts microtubules

17

## 18 **Abstract**

19 *Salmonella* effector protein SseJ is secreted by *Salmonella* into the host cell  
20 cytoplasm where it can then modify host cell processes. Whilst host cell small  
21 GTPase RhoA has previously been shown to activate the acyl-transferase  
22 activity of SseJ we show here an un-described effect of SseJ protein  
23 production upon microtubule dynamism. SseJ prevents microtubule collapse  
24 and this is independent of SseJ's acyl-transferase activity. We speculate that  
25 the effects of SseJ on microtubules would be mediated *via* its known  
26 interactions with the small GTPases of the Rho family.

## 27 **Introduction**

28 *Salmonellae* are gram-negative bacteria that can infect a wide range of  
29 hosts and in humans can cause diseases such as typhoid fever and  
30 gastroenteritis. There are ~2600 recognized *Salmonella* serovars of which  
31 over half are represented by *S. enterica* subspecies *enterica* (*S. enterica*  
32 subspecies I), constituting 99% of human  
33 clinical *Salmonella* infections. *Salmonella enterica* serovar Typhimurium (*S.*  
34 Typhimurium; the cause of gastroenteritis) uses two type III secretion systems  
35 (T3SS) to translocate pathogen effector proteins directly into the host cell's  
36 cytoplasm. (reviewed by [1]). The T3SS encoded by *Salmonella* pathogenicity  
37 island-1 (SPI-1; T3SS-1) is mostly active when extracellular *Salmonella* come  
38 into contact with a host cell and allows effector proteins to be translocated  
39 directly into the cell cytoplasm and causes the bacteria to be actively  
40 phagocytosed. Another T3SS encoded by *Salmonella* pathogenicity island-2  
41 (SPI-2; T3SS-2) enables the bacteria to multiply intracellularly in a *Salmonella*

42 containing vacuole (SCV) by allowing further effector proteins to be  
43 translocated directly from the *Salmonella* (through the phagosomal  
44 membrane) into the host cell cytoplasm. It is unclear precisely how  
45 *Salmonella* uses its multiple T3SS effector proteins to survive intracellularly  
46 but theories range from delaying fusion with the degradative organelle the  
47 lysosome [2], though the role of the T3SS in this process is contested [3], to  
48 preventing the delivery of lysosomal hydrolases to the *Salmonella*-containing  
49 phagosomal compartment by altering mannose 6-phosphate receptor  
50 trafficking [4]. Only a finite number of intracellular membrane trafficking and  
51 signalling events can be manipulated by a pathogen and hence successful  
52 intracellular pathogens are often found to target the same host cell molecules,  
53 for instance phosphoinositides are targeted by both *Salmonellae* and  
54 *Mycobacteria* [5, 6]. Understanding how *Salmonella* survives intracellularly not  
55 only provides information about *Salmonella* pathogenesis but potentially what  
56 processes may also be targeted by other intracellular pathogens.

57         To understand the role of *Salmonella* T3SS effector proteins in the flow  
58 of membranes to the lysosome a rapid screen was undertaken in  
59 *Saccharomyces cerevisiae* (*S. cerevisiae*). Membrane trafficking events are  
60 conserved between yeast and mammalian cells. Therefore, yeast can be used  
61 to rapidly identify any *Salmonella* proteins that alter membrane traffic to the  
62 yeast vacuole, the equivalent of the mammalian lysosome. The screen  
63 identified the *Salmonella* virulence protein SseJ and subsequently we show a  
64 previously un-described effect of this protein on the stability of host cell  
65 microtubules. Microtubules are required for phagosome fusion [7-9] and by  
66 promoting a network of stable microtubules this can aid in phagosome fusion

67 with endocytic organelles enabling nutrients to be delivered to the  
68 phagosomal lumen, promoting bacterial replication.

69

## 70 **Results**

### 71 **SseJ production causes membrane trafficking defects**

72 To identify *Salmonella* proteins that can disrupt intracellular membrane  
73 trafficking, a genomic library from *Salmonella* was generated and the DNA  
74 inserted into a yeast expression vector. *S. cerevisiae* were then transformed  
75 with the plasmid library and colonies screened for a defect in the delivery of  
76 the vacuolar hydrolase, carboxypeptidase-Y (CPY), to the yeast vacuole. If  
77 there is disruption of CPY delivery to the vacuole then CPY is secreted. We  
78 assayed the secretion of a CPY-invertase fusion protein that oxidises an  
79 applied solution of o-diansidine to a brown precipitate [10]. This approach has  
80 been successfully employed to identify effector proteins of *Legionella*  
81 *pneumophila* [11] and *Mycobacterium tuberculosis* [12] that interfere with  
82 yeast membrane trafficking. Yeast transformed with the plasmid library were  
83 screened for CPY-Inv secretion and 8 yeast clones were found to have CPY-  
84 Inv secretion in a plasmid dependent manner. One of the clones identified a  
85 6kb fragment of *Salmonella* chromosomal DNA containing 1 partial open  
86 reading frame (ORF) and 6 complete ORFs (Fig 1A). All of the *Salmonella*  
87 genes identified in the plasmid, were cloned and expressed individually in  
88 yeast and re-assayed for CPY secretion. Qualitative CPY-Inv secretion on  
89 agar plates showed that SseJ caused CPY secretion, though we did not  
90 analyse the protein production levels of the other 5 proteins. (Fig. 1B).

91 Quantitative CPY-Inv secretion from yeast in liquid culture demonstrated that  
92 SseJ dependent CPY-Inv secretion was equivalent to that in yeast lacking the  
93 CPY receptor, VPS10 ( $\Delta$ VPS10; fig. 1C). There are numerous intermediate  
94 vesicles involved in delivery of CPY to the vacuole and the retrograde  
95 trafficking of the VPS10 receptor. When CPY is secreted, due to a trafficking  
96 defect, it is possible to examine the phenotype of the yeast vacuole and in  
97 some cases determine which part of the trafficking step of CPY from the Golgi  
98 to the vacuole is disrupted [13, 14]. Using the membrane dye FM4-64 to label  
99 the yeast vacuole in yeast expressing SseJ, no differences in the morphology  
100 of the vacuole were seen compared to wild-type yeast (fig. 1D). These data  
101 indicated that SseJ alone can cause a membrane trafficking defect in yeast.

#### 102 **SseJ production re-distributes late endocytic organelles**

103 SseJ is one of several virulence proteins secreted by *Salmonella*'s T3SSs into  
104 the host's cytoplasm directly from the bacteria [15]. *Salmonella* strains lacking  
105 SseJ are attenuated in replication [16-19] indicating that SseJ is crucial for  
106 bacterial intracellular replication. SseJ was then expressed in mammalian  
107 cells. In this case, we used Normal Rat Kidney (NRK) cells since they show  
108 good spatial resolution between endocytic vesicles and in particular between  
109 late endosomes and lysosomes. Late-endocytic organelles are poorly  
110 resolved by light microscopy in HeLa cells, which are often used for  
111 *Salmonella* infection studies. Constitutive protein production of SseJ was  
112 found to cause cell death so myc-tagged SseJ (myc-SseJ) was expressed  
113 under the control of a metallothionein promoter allowing for inducible *sseJ*  
114 expression upon the addition of cadmium. Immunofluorescence demonstrated  
115 that myc-SseJ localises to lysosomes (Fig. 2A) as has previously been

116 reported [19]). Moreover there was a dramatic re-distribution of late endocytic  
117 organelles with both late endosomes and lysosomes becoming less peri-  
118 nuclear and more peripherally distributed (Fig. 2B). The *trans*-Golgi marker  
119 TGN38 was observed to occupy a larger area of the cell (Fig. 2B), but in  
120 general cells were flatter with an increased surface area (on average the cell  
121 surface area went from 591 $\mu\text{m}^2$  to 2,057 $\mu\text{m}^2$  upon SseJ expression). Ectopic  
122 SseJ protein production can cause globular membranous compartments  
123 (GMCs) [19] and indeed when *sseJ* expression was induced for 24 h,  
124 lysosomes were seen to aggregate as observed by LGP120 (rat equivalent of  
125 LAMP1) staining (Fig. 2C). The metallothionein promoter regulating *sseJ*  
126 expression is slightly leaky due to the presence of trace amounts of heavy  
127 metals in the tissue culture media, which explains why the lysosomes are  
128 partially aggregated in transfected cells before cadmium addition (Fig. 2C  
129 panel b). The re-distribution of organelles is observed when the cytoskeleton  
130 is perturbed [20] and indeed when the microtubule polymerisation inhibitor  
131 nocodazole was added to cells, late endocytic organelles re-distributed in a  
132 manner similar to that observed with SseJ expression (Fig. 2D).

### 133 **SseJ alters microtubule dynamics**

134 To assess whether the re-distribution of organelles was related to changes to  
135 the cytoskeleton the microtubules were visualised in cells expressing SseJ or  
136 a mutant SseJ (SseJ S151A). SseJ has homology to the GDSL-like lipolytic  
137 enzyme family [21] and shows deacylase, phospholipase and  
138 glycerophospholipid-cholesterol acyltransferase (GCAT) activity [22-24].  
139 Ser151 in SseJ is the middle serine in a GDSL motif, which is present in  
140 GCAT enzymes and mutation of this residue reduces SseJ's deacylase

141 activity by 5 fold [22]. SseJ-S151A still localises to the *Salmonella* containing  
142 vacuole and *Salmonella* induced filaments (Sifs) [25] are still visible in a SseJ-  
143 S151A mutant strain but the bacteria show reduced virulence [22]. The  
144 microtubules, in both WT and mutant-SseJ expressing cells, became  
145 disorganised with no clear microtubule organising centre (MTOC; Fig. 3A). In  
146 J774.2 macrophages the majority of cells don't have clear microtubules  
147 emanating from a MTOC unless they have flattened out on the culture vessel  
148 surface (Fig. 3B). Co-cultures of bacteria and J774.2 macrophages causes  
149 the macrophages to flatten out and under these conditions the microtubule  
150 network becomes more visible. However, a loss of organised microtubules,  
151 emanating from a clearly defined MTOC, was seen in mouse macrophages  
152 infected with WT *Salmonella* but not in cells infected with  $\Delta$ sseJ *Salmonella*  
153 (Fig. 3B). Typically,  $\Delta$ sseJ *Salmonella* induced a four-fold increase in visible  
154 microtubules emanating from the MTOC compared to control cells, but WT  
155 *Salmonella* only induced a two-fold increase (Fig. 3B). Unlike nocodazole that  
156 completely disrupts tubulin polymers (Fig. 2D), cells expressing SseJ still  
157 show some tubulin polymers albeit in a dis-organised manner. Long-lived,  
158 stable microtubules are de-tyrosinated, resulting in the exposure of a  
159 glutamate residue (Glu-tubulin), and acetylated [26, 27]. Cells were then  
160 examined for the presence of Glu-tubulin (Fig. 3C). In cells expressing both  
161 WT and mutant SseJ protein there was a reduction in Glu-tubulin  
162 immunolabelling compared to control cells. Furthermore, there was a  
163 reduction in acetylated-tubulin (Fig. 3D) in WT and mutant sseJ expressing  
164 cells. The reduction in acetylated-tubulin corresponded to the time of induction  
165 of sseJ expression (Fig. 3E). SseJ protein production was induced with 10 $\mu$ M



166 cadmium and the metal can alter the cytoskeleton [28, 29] but we saw no  
167 effect of cadmium on the cytoskeleton in NRK cells without *ssej* expression  
168 (all control cells in figure 3 are in the presence of 10 $\mu$ M cadmium chloride).  
169 Together these data suggested that long-lived microtubules had been de-  
170 stabilised in cells expressing SseJ, but some un-organised microtubules could  
171 still be observed. When cells were transfected with a plasmid encoding GFP-  
172 CLIP-170, a protein that binds to the growing ends of microtubules, and  
173 visualised by live cell microscopy, no CLIP-170 movement could be observed  
174 in cells expressing SseJ (supplemental movie) compared to control cells  
175 (supplemental movie). Similar data were obtained with EB3-tdTomato,  
176 another microtubule plus-end binding protein, and single images of EB3-  
177 tdTomato transfected cells show the EB3 on the end of microtubules in control  
178 cells but no visible incorporation of the EB3 onto microtubules in cells  
179 expressing SseJ (Fig 3F). So whilst there was a reduction in long-lived  
180 microtubules, as assessed by Glu-tubulin and acetylated-tubulin, there was no  
181 dynamism in the remaining microtubules.

## 182 **SseJ binds both RhoA and RhoC**

183 Rho proteins are small GTPases that are primarily associated with modifying  
184 the actin cytoskeleton, but they can effect cell polarity and microtubules [30].  
185 SseJ can interact with both RhoA or RhoC [31, 32], with GTP-bound RhoA  
186 activating SseJ's lipase activity [32]. SseJ has only previously been shown to  
187 bind RhoA or RhoC separately. Large scale immunoprecipitations of SseJ  
188 from cells overexpressing SseJ identified both RhoA and RhoC having bound  
189 to SseJ under experimental conditions where the GTPases were in their GDP-  
190 bound form (Fig. 4A), with WT and SseJ-S151A binding Rho proteins with

191 equal ability (Fig. 4B). These experiments indicate that SseJ can bind either  
192 RhoA or RhoC in the presence of each other when neither protein is in a  
193 limiting amount. Although we have no evidence, it is unlikely that SseJ is  
194 binding both RhoA and RhoC simultaneously. Using an ELISA we found, as  
195 has been reported [32], that SseJ did not increase the levels of activated  
196 (GTP-bound) RhoA (Fig. 4C).

## 197 **Discussion**

198 In this study, we aimed to understand how *Salmonella* can survive  
199 intracellularly by uncovering *Salmonella* effector molecules that can  
200 manipulate membrane trafficking events. Manipulation of membrane traffic  
201 may disrupt late-organelle biogenesis, including lysosomes, and therefore  
202 provide conditions that enable the bacteria to replicate. We hypothesised that  
203 a *Salmonella* T3SS effector molecule may manipulate membrane trafficking in  
204 yeast to the same extent as mammalian cells given that the delivery of  
205 molecules to the vacuole/lysosome are conserved. Using an unbiased screen  
206 we identified SseJ, which is a T3SS effector protein, that caused a membrane  
207 trafficking defect in yeast (Fig. 1). This is the first demonstration that SseJ  
208 causes changes to membrane trafficking in eukaryotes. The powerful yeast  
209 screen led us to examine the distribution of organelles in mammalian cells,  
210 expecting them to be perturbed. Indeed, organelles no longer localised to the  
211 MTOC (Fig. 2) and this observation could be related to changes to the  
212 microtubules (Fig. 3). We further showed that SseJ can bind to both RhoA  
213 and RhoC and whilst others have shown that RhoA can regulate the GCAT  
214 activity of SseJ [32] this is the first report to prove the hypothesis that SseJ  
215 alters the cytoskeleton [33].

216           How might SseJ alter the cytoskeleton? Whilst Rho proteins are well  
217 known to alter the actin cytoskeleton they can also alter the stability of  
218 microtubules via Diaphanous-related formins (DRFs) [34]. RhoA-mDia1/2 can  
219 stimulate microtubule stabilisation with an increase in Glu-tubulin, precisely  
220 how this is achieved is unknown, and it is possible that if SseJ recruits active  
221 Rho proteins to the lysosome then the RhoA-mDia1/2 balance may be  
222 disrupted leading to changes in the microtubules. Whilst we did not observe  
223 an increase in Glu-tubulin we did see static microtubules. Although the  
224 binding of SseJ to RhoA or RhoC has been documented, our data show for  
225 the first time that SseJ can bind RhoA or RhoC when both proteins are  
226 present and neither are in limited amounts i.e. SseJ does not preferentially  
227 bind RhoA and then RhoC (Fig 4). This does raise the possibility that SseJ  
228 may have differential effects through both RhoA and RhoC, with differences  
229 between RhoA, RhoB and RhoC well documented [35]. So whilst RhoA-GTP  
230 can stimulate the GCAT activity of SseJ [32], the binding of RhoC to SseJ  
231 may affect the microtubules. RhoC is reported to have a higher affinity for the  
232 kinases Rho-associated coiled-coil containing kinases (ROCK) and Citron  
233 kinase compared to RhoA [35]. MAP2/Tau proteins stabilise microtubules and  
234 inhibit depolymerisation (reviewed by [36]), an effect seen in SseJ expressing  
235 cells, and MAP2/Tau proteins can be phosphorylated by numerous kinases  
236 including ROCK [35, 37]. The effects of MAP2/Tau phosphorylation are yet to  
237 be determined, but there is a precedence for microtubule regulation by Rho  
238 proteins via DRFs and kinases such as ROCK [38]. Expression of *sseJ* before  
239 *Salmonella* infection reduces Sif formation [19], which can be explained by the  
240 fact that a dynamic cytoskeleton is required for phagosome maturation [39].

241 Additionally, whereas SseJ-S151A has reduced GCAT activity [22] the effects  
242 on the microtubules are still seen in the S151A mutant suggesting that the  
243 GCAT activity is separate from the microtubule effect, though we can't rule out  
244 that there is still enough residual GCAT activity in cells over-expressing *sseJ*.

245 SseJ has been shown to interact indirectly with another T3SS effector  
246 protein, SifA [31].  $\Delta$ *sifA* mutants escape the phagosomal vacuole but not if a  
247 double *sifA sseJ* mutant is made, implying that loss of the integrity of the  
248 phagosomal membrane is dependent on SseJ [19]. SifA and SseJ are  
249 sufficient to cause endosome tubulation [31] and certainly SifA is required for  
250 endosome tubulation [40, 41]. With SifA found to bind to RhoA, and SKIP,  
251 which is a kinesin binding protein, it was hypothesised that RhoA, SseJ, SifA  
252 and SKIP regulates endosome tubulation along microtubules [31]. However,  
253 studies have shown that  $\Delta$ *sseJ Salmonella* show endosomal tubulation  
254 implying that SseJ is dispensable for endosome tubulation in a background  
255 where all the other secreted effector proteins are expressed [42, 43].

256 *Salmonella* induced endosomal tubules or *Salmonella* induced  
257 filaments (Sifs) are initially dynamic but become stabilised (>8h after cell  
258 infection; [42] and this stabilisation could correspond to the changes that we  
259 see in the dynamics of the microtubules, given that SseJ is secreted from  
260 *Salmonella* within 4 h [17]. It is has been known for a long time that  
261 lysosomes can form tubules [44, 45] and that microtubules regulate the  
262 distribution of lysosomes [46] and their tubular morphology [7]. Although SseJ  
263 is dispensable for the formation of Sifs in infected cells, SseJ may aid in  
264 stabilising the Sifs that do form.. Why would this be advantageous to the  
265 *Salmonella*? Endosome fusion and delivery of endocytosed material to

266 lysosomes can occur at the end of lysosome tubules [47] and the curvature of  
267 the membrane at the tip of a tubule is likely to be more fusogenic with  
268 endocytic vesicles compared to a larger, more-rounded phagosomal  
269 membrane [48]. By reducing microtubule de-polymerisation this allows  
270 *Salmonella* to promote tubular lysosomes (endosomal tubules), in conjunction  
271 with other proteins such as SifA, increasing fusion events with endosomal  
272 vesicles carrying in nutrients from the extracellular environment. Rho  
273 GTPases are a common target of bacterial pathogens [49, 50] and further  
274 work is required to determine whether SseJ's effect on cellular microtubules is  
275 mediated through RhoA or RhoC.

## 276 **Materials and Methods**

### 277 **Reagents and Antibodies**

278 Chemical reagents were of laboratory grade. Anti-c-myc (9E10) antibodies  
279 were purified from 9E10 hybridoma tissue culture supernatants  
280 (Developmental Studies Hybridoma Bank). Anti rat LGP110 (580), anti-mouse  
281 cation-independent mannose 6-phosphate receptor (MPR; 1001) and anti-rat  
282 TGN38 (2F7.1) were kind gifts from J. P. Luzio (University of Cambridge, UK).  
283 Anti  $\alpha$ -tubulin (T-9026) was from Sigma, anti-glu-tubulin was from Synaptic  
284 Systems, anti-acetylated tubulin (D20G3), rabbit monoclonal anti-RhoA  
285 (67B9) and anti-RhoC (D40E4) were from Cell Signalling.

### 286 **Yeast Strains**

287 BHY10 and BHY11 haploid yeast strains expressing CPY-Inv [51] and BHY10  
288  $\Delta$ VPS10::TRP1 were a kind gift from Dr. M. Seaman (University of

289 Cambridge). For the screen BHY10 and BHY11 were mated on YPD agar  
290 plates, diploid yeast (BHY12) picked from SC–Lys,-Ade plates and then  
291 maintained on YPD agar plates.

## 292 ***Salmonella* genomic library generation**

293 Chromosomal DNA was isolated from stationary phase *Salmonella*  
294 Typhimurium strain 14028 [52]. DNA was partially digested with Sau3AI for 1h  
295 at 37°C. DNA was electrophoresed on a gel, and the region corresponding to  
296 ≈0.8-5 kb was excised and the DNA purified. pVT-100 U [53] a gift from Dr. K.  
297 Bowers (UCL, UK), was linearised with BamHI and then de-phosphorylated  
298 using calf intestinal phosphatase. DNA was ligated into linearised pVT-100U  
299 using T4 DNA ligase and transformed into NEB 10-beta competent *E.*  
300 *coli* (High Efficiency). Ampicillin-resistant colonies (≈0.5x10<sup>6</sup>) were scraped,  
301 and plasmid DNA prepared (Qiagen midiprep).

## 302 **Constructs**

303 SseJ was cloned from *S. Typhimurium* DNA by PCR. Primers were used to  
304 append a myc-tag to the SseJ PCR product along with 15bp regions of  
305 homology to the destination vector to allow for homologous recombination  
306 using In-Fusion cloning (Invitrogen). The myc-tagged SseJ DNA was inserted  
307 into the HindIII restriction enzyme site of the ΔpMEP4 vector [54] by  
308 homologous recombination. The S151A mutant was made by QuikChange  
309 site-directed mutagenesis (Stratagene) of the myc-SseJ construct as per the  
310 manufacturer's instructions.

311

### 312 **Invertase (Inv) assays**

313 The *Salmonella* plasmid library was transformed into BHY12 yeast [55], and  
314 transformants were plated on synthetic complete medium without uracil (SC-  
315 Ura) plates with 2% (w/v) fructose. Carboxypeptidase-Y-invertase (CPY-Inv)  
316 assay, both quantitatively and qualitatively, is based on previous  
317 methodologies [10].

### 318 **FM4-64 Staining**

319 1ml of log-phase yeast were pelleted and then resuspended in 50 $\mu$ l YPD  
320 medium containing 40 $\mu$ M FM 4-64 (Molecular Probes). Yeast were incubated  
321 at 30°C for 15 min before the yeast were pelleted and washed with YPD  
322 media. Yeast in fresh YPD were then incubated for 30 min at 30°C. Yeast  
323 were pelleted and then resuspended in 1ml of H<sub>2</sub>O and then aliquots directly  
324 visualised by confocal microscopy.

### 325 **Tissue culture and cell transfection**

326 All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM),  
327 supplemented with 10% (v/v) FCS, 100U/L penicillin, 100mg/L streptomycin  
328 and 2mM L-glutamine, in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were  
329 transfected with plasmid DNA using Fugene 6 (Roche Diagnostics) as per the  
330 manufacturer's instructions.  $\Delta$ pMEP4 transfected cells were selected with  
331 media containing 0.2 mg/ml hygromycin to generate a stable population of  
332 transfected cells and individual clones were selected and assessed for SseJ  
333 protein production. SseJ production was induced with 10  $\mu$ M CdCl<sub>2</sub> for 16-24h  
334 before analyses.

335 **J774.2 *Salmonella* infection.**

336 J774.2 cells were seeded onto glass coverslips and cultured for 48 h in  
337 antibiotic-free DMEM medium supplemented with 10 % (v/v) FBS (heat  
338 inactivated to 56 °C for 30 min) and 2 mM glutamine. *Salmonellae* (WT and  
339  $\Delta$ SseJ *Salmonella enterica* serovar *Typhimurium* strain 12023 were a kind gift  
340 from Prof. David Holden, Imperial College London). were cultured overnight in  
341 LB media with shaking at 30 °C. An appropriate number of bacteria were  
342 taken to infect J774.2 cells at an MOI (multiplicity of infection) of 10 and  
343 resuspended in PBS. Bacteria were centrifuged onto cells at 80 x g for 5 min  
344 and incubated for 1 h at 37 °C to allow phagocytosis of bacteria. Monolayers  
345 were rinsed 3 times with DMEM to remove unbound bacteria, and the media  
346 replaced with DMEM containing 150 µg/ml gentamycin to kill extracellular  
347 bacteria. The cells were cultured for a further hour, and washed with PBS.  
348 The media was then replaced with DMEM containing 10 µg/ml gentamycin,  
349 and cells cultured for 24 h to allow intracellular bacteria to grow. Cells were  
350 fixed with 4 % formaldehyde in PBS for 20 min at room temperature and then  
351 processed for immunofluorescence.

352 **Immunofluorescence**

353 Cells were fixed with 4 % (w/v) formaldehyde in PBS for 20 min at 20°C. Cells  
354 to be immunolabelled for microtubules were rinsed with microtubule stabilising  
355 buffer (MTSB; 80mM PIPES, pH 6.8, 1mM MgCl<sub>2</sub>, 4mM EGTA) then  
356 incubated in MTSB containing 0.05% (w/v) saponin (Sigma S-4521) for 1 min  
357 then fixed with 2% (w/v) formaldehyde, 0.05% (w/v) glutaldehyde in MTSB for  
358 20 min. Cells immunolabelled for Glu-tubulin were fixed with -20°C MeOH for



359 5 min at -20°C. All fixed cells were incubated for 10 min in 50 mM NH<sub>4</sub>Cl in  
360 PBS followed by 10 min in 0.2 % (w/v) BSA in PBS containing 0.5% (w/v)  
361 saponin (PBS-BS). Cells were immunolabelled with primary antibodies in  
362 PBS-BS for 1h at 20°C. Cells were rinsed 3 x 5 min with PBS-BS and then  
363 incubated with fluorescent secondary-antibodies in PBS-BS for 30 min at  
364 20°C. Cells were rinsed 3 x 5 min with PBS-BS before being mounted in  
365 Mowiol. Fluorescence was imaged using a Zeiss LSM510 confocal  
366 microscope. All images are maximum intensity z-projections unless otherwise  
367 stated.

### 368 **Cell lysates**

369 Lysates were generated by rinsing cells with PBS and then scraping cells into  
370 ice cold lysis buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 0.5 %  
371 (v/v) NP-40). Lysates were left on ice for 10 min before removal of detergent  
372 insoluble material by centrifugation (16,400 g, 10 min, 4°C).

### 373 **Immunoprecipitation**

374 9E10 antibody was coupled to Amino Link Plus resin (Pierce) following the  
375 manufacturer's instructions. Small scale immunoprecipitations used 20 µl of  
376 resin and 250µg of cell lysate and samples were incubated for 2 h at 4°C with  
377 rotation. Resins were washed 3 x with lysis buffer and immunoprecipitated  
378 proteins eluted using IgG gentle elution buffer (Pierce) and analysed by SDS-  
379 PAGE. Large scale immunoprecipitations used 12 x T75 flasks and 2ml of  
380 anti-myc resin.

### 381 **Mass spectroscopy**

382 Proteins in gel bands were reduced with DTT and alkylated with  
383 iodoacetamide before digestion with modified porcine trypsin  
384 (Promega). Digests were dissolved in 4-hydroxy- $\alpha$ -cyano-cinnamic acid and  
385 analysed by positive-ion MALDI-MS/MS using a Bruker ultraflex III. Spectra  
386 were submitted to Mascot MS/MS ions search against the NCBI database.

### 387 **RhoA activity assays**

388 Active RhoA in cell lysates was assessed by ELISA using a RhoA activity  
389 assay (RhoA G-LISA; Cytoskeleton, Inc) as per the manufacturer's  
390 instructions.

### 391 **Live cell imaging**

392 NRK cells (WT) or expressing SseJ were transfected with either GFP-  
393 CLIP170 (kind gift of Folma Buss, University of Cambridge) or EB3-tdTomato  
394 (a kind gift from Dr Anne Straube, University of Warwick) and 24 hours later  
395 imaged on an Andor Spinning Disc Confocal Microscope. Images were  
396 collected with 200ms exposures and a 800ms delay between exposures,  
397 giving 1 frame per second.

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403 **Acknowledgements**

404 We thank Gareth Evans, Nia Bryant, Jonathan Bennett and Nathalie Signoret  
405 for helpful advice and for reading the manuscript.

406 **Author Contributions**

407 S.A.R. and M.H. provided technical support and carried out some of the  
408 experiments. A.A.D carried out the proteomic identification of proteins. P.R.P  
409 designed and carried out the majority of the research and wrote the  
410 manuscript.

411

412

413 **Figure Legends**

414 **Figure 1. Expression of *ssej* causes CPY-Inv to be mis-sorted in**  
415 ***Saccharomyces cerevisiae*.** (A) Fragment of the *Salmonella* chromosome  
416 inserted into the yeast expression vector causing CPY-Inv secretion. (B)  
417 Qualitative CPY-Inv secretion in yeast expressing individual *Salmonella* genes  
418 identified in (A). Negative control yeast (ctrl) contain just the cloning vector  
419 (pVT-100U) and positive control yeast lack the receptor VPS10 for CPY  
420 ( $\Delta$ VPS10). (C) Quantitative CPY-Inv secretion in yeast expressing *Salmonella*  
421 genes. Controls as in (B). Data are from n=3-9 (number of experiments for  
422 each condition in parentheses above each bar) and are mean  $\pm$  S.D.  
423 \*P<0.001 SseJ c.f. Ctrl (P>0.05 SseJ c.f.  $\Delta$ VPS10). (D) Fluorescence  
424 visualisation of the yeast vacuole in wild type yeast (WT) transformed with  
425 vector (pVT-100U) alone or SseJ in pVT-100U (SseJ). Top panels DIC and  
426 bottom panels FM 4-64 fluorescence. Scale bar = 10  $\mu$ m.

427 **Figure 2. Re-distribution of late endocytic organelles in cells expressing**  
428 **SseJ.** (A) NRK cells expressing myc-SseJ were double labelled with anti-myc  
429 (a) and anti-lysosome glycoprotein 110 (Lgp110; b) followed by fluorescently  
430 labelled secondary antibodies. Panel c is the merged image of panels a and b,  
431 co-localisation is shown by yellow. (B) Control (Ctrl) NRK cells or NRK cells  
432 expressing myc-SseJ (myc-SseJ) were immuno-labelled for the mannose 6-  
433 phosphate receptor (MPR), Lgp110 and *trans*-Golgi network 38 (TGN38)  
434 followed by fluorescently-labelled secondary antibodies to visualise the late  
435 endosomes, lysosomes and *trans*-Golgi network respectively. (C) Aggregation  
436 of Lgp110 in NRK cells expressing SseJ for 24h (a). Quantification of cells

437 showing aggregated lysosomes after induction of SseJ production with  
438 cadmium (Cd) (b). Expression of myc-SseJ protein +/- Cd is shown by the  
439 western blot insert (b). (D) NRK cells were immunolabelled for microtubules  
440 ( $\alpha$ -tubulin; a,d), lysosomes (lgp120;b,e) and late endosomes (cation-  
441 independent mannose 6-phosphate receptor; c,f) in control cells (ctrl) or after  
442 cells had been treated with 10 $\mu$ M nocodazole for 1h. Scale bars represent  
443 10 $\mu$ m.

444 **Figure 3. Microtubules are disrupted in cells expressing SseJ.** (A) Control  
445 (Ctrl) NRK cells and cells expressing myc-SseJ (SseJ) or myc-SseJ-S151A  
446 (S151A) were fixed and the microtubules visualised using anti  $\alpha$ -tubulin  
447 antibodies and fluorescently-labelled secondary antibodies. Bars = 10 $\mu$ m. (B)  
448 J774.2 mouse macrophages were either uninfected (Ctrl) or infected with WT  
449 or  $\Delta$ sseJ *Salmonella* Typhimurium for 24h before fixing. The DNA (blue) was  
450 visualised using DAPI and the microtubules (red) were visualised as in A.  
451 Bars = 20 $\mu$ m. Quantification of the number of cells showing an organised  
452 microtubule network under each condition is shown (n=1, scoring 100 cells  
453 per condition). (C) Cells as in A were fixed and de-tyrosinated  $\alpha$ -tubulin (Glu-  
454 tubulin) visualised by immunolabelling using anti Glu-tubulin antibodies and  
455 fluorescently-labelled secondary antibodies. Bars = 10 $\mu$ m. (D) Cells as in A  
456 were lysed and lysates immunoblotted for acetylated- $\alpha$ -tubulin (Ac-tubulin)  
457 and  $\alpha$ -tubulin. (E) myc-SseJ production was induced in NRK cells up to 24h.  
458 Lysates were generated and western blotted for myc-SseJ, acetylated-tubulin  
459 and Rho (pan specific). (F) NRK cells (Ctrl) and those expressing sseJ (SseJ)  
460 were transfected with a plasmid encoding EB3-tdTomato. EB3-tdTomato was

461 visualised live, 24h later, on a spinning disc confocal microscope. Images  
462 represent a single time frame. Bars = 10µm.

463

464 **Figure 4. SseJ binds GTPases RhoA and RhoC.** (A) Anti-myc antibody was  
465 covalently attached to sepharose and myc-SseJ was immunoprecipitated from  
466 control (Ctrl) NRK cells or NRK cells expressing myc-SseJ (myc-SseJ).  
467 Proteins bound to the beads were eluted and subjected to SDS-PAGE and  
468 the gel stained with coomassie (shown). SseJ is indicated by an arrowhead. A  
469 band at ≈21kDa specifically found in the SseJ immunoprecipitation was  
470 excised and sequenced by mass spectroscopy and identified both RhoA and  
471 RhoC. Peptides identified are shown by the insert with peptides common to  
472 both RhoA and RhoC shown in bold, peptides unique to RhoA shown in blue  
473 and peptides unique to RhoC shown by red. Only a single peptide was unique  
474 to RhoC (highlighted by an asterisk). (B) Experiments as shown in A,  
475 including cells expressing myc-SseJ(S151A), were repeated and western  
476 blotted for myc, RhoA and RhoC. Western blots show 1/10<sup>th</sup> of the input  
477 before and after the immunoprecipitation and the total eluate from the  
478 immunoprecipitations. (C) The activity of RhoA was measured by ELISA, on  
479 extracts from control cells and cells expressing myc-SseJ or myc-SseJ  
480 (S151A) mutant. Data are means ± SD, n=8.

481

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701

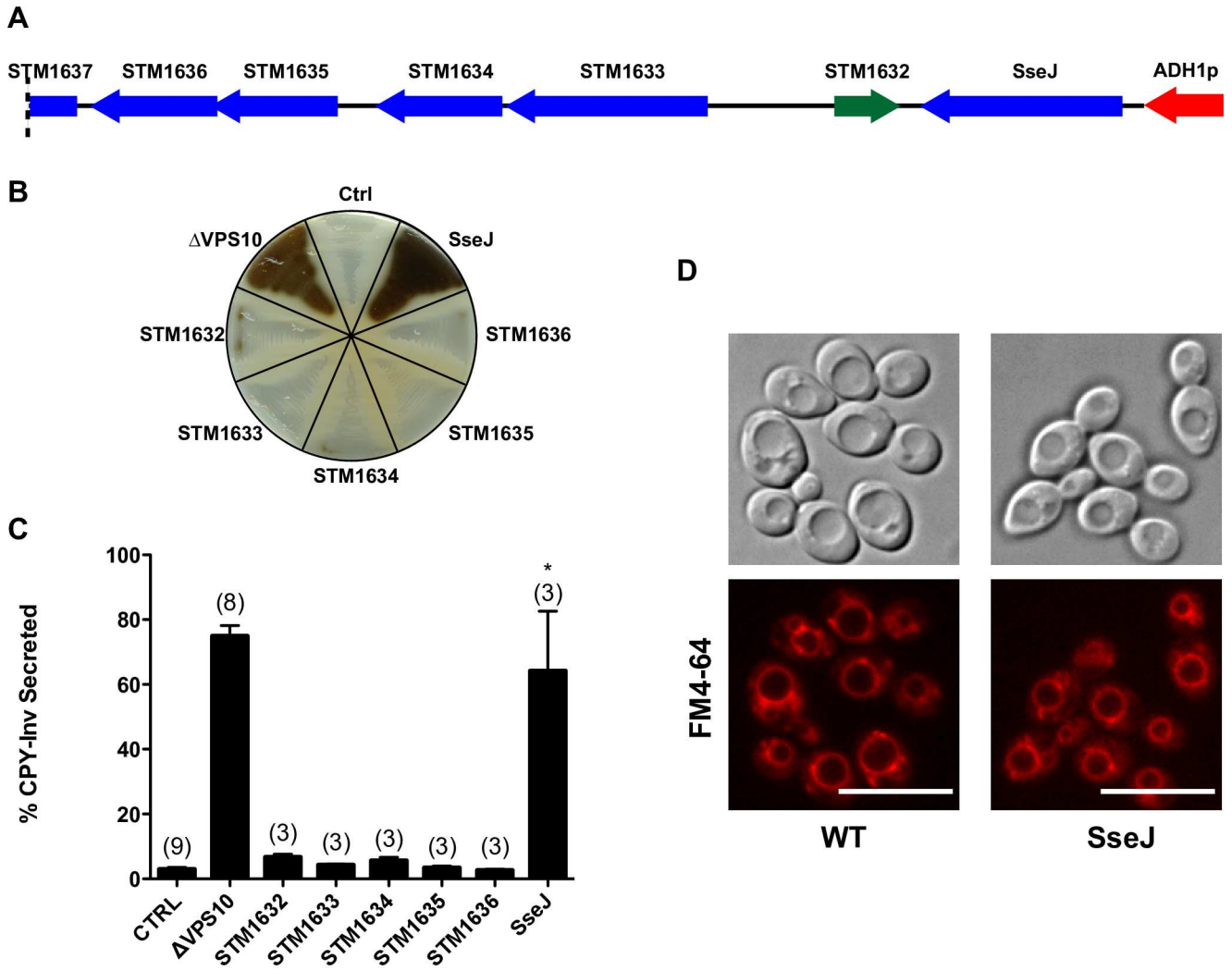


Fig 1

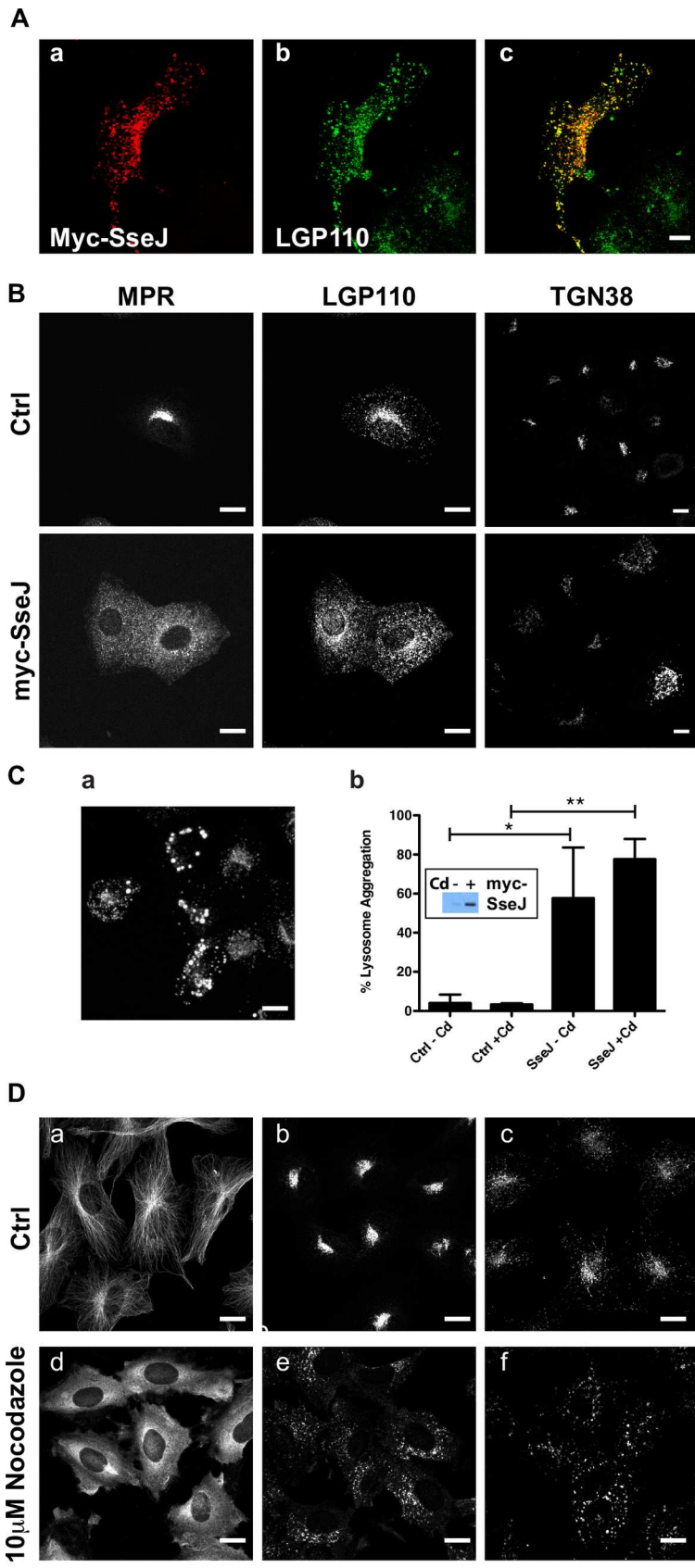


Fig 2

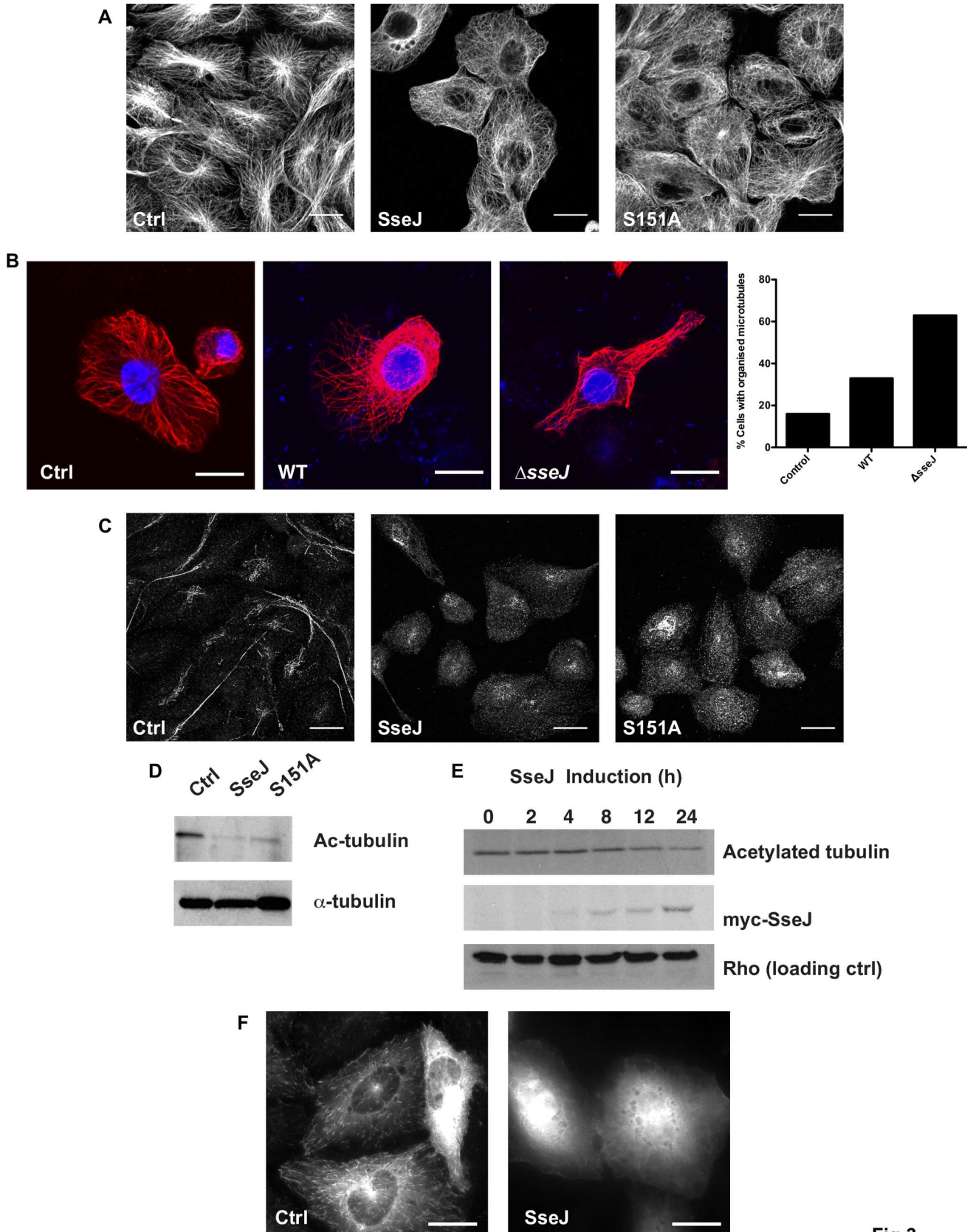
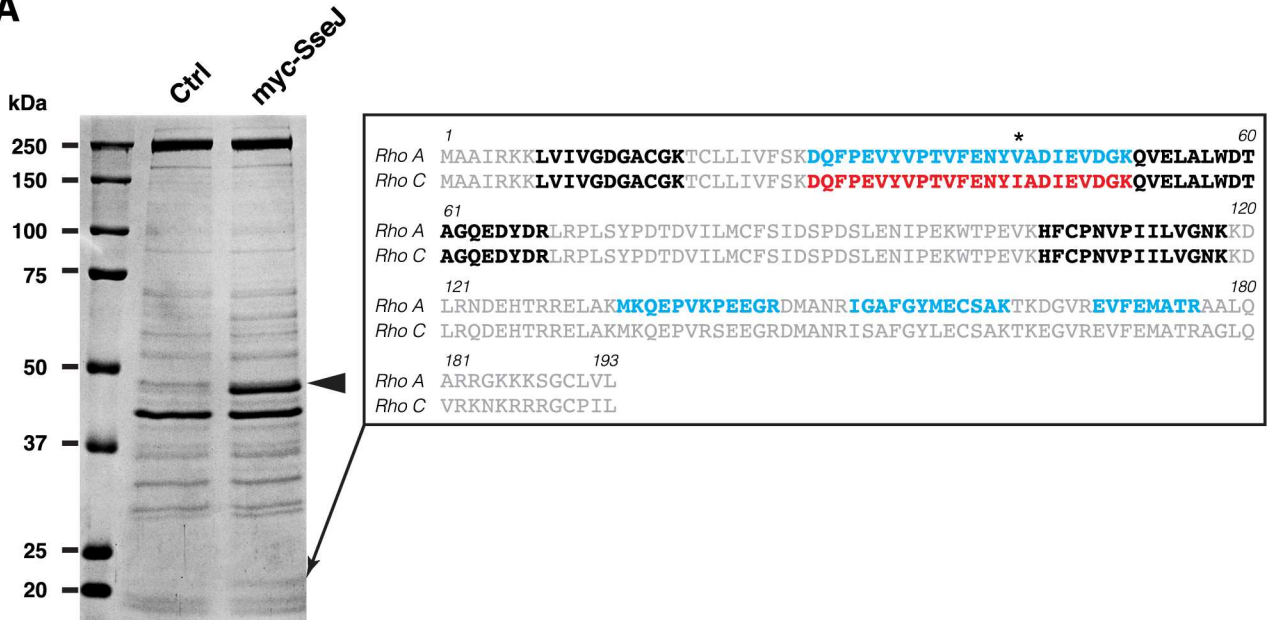


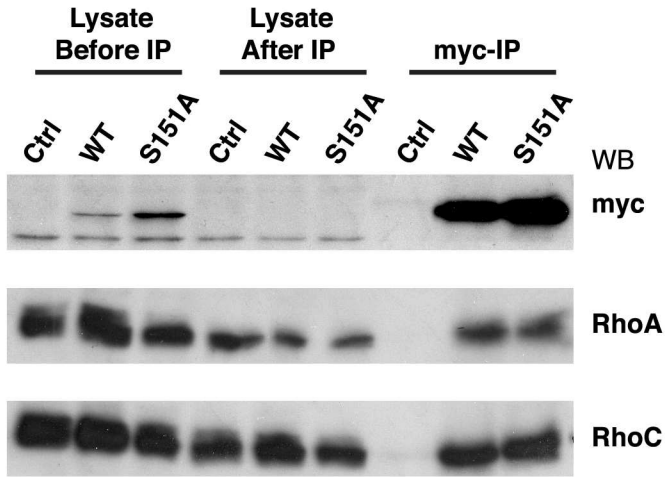
Fig 3



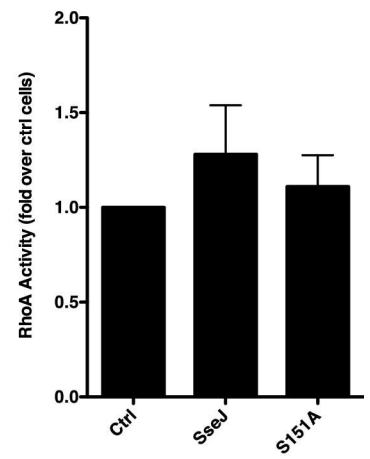
**A**



**B**



**C**



**Fig 4**