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

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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

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

Results

SseJ production causes membrane trafficking defects

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

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

SseJ alters microtubule dynamics

To assess whether the re-distribution of organelles was related to changes to the cytoskeleton the microtubules were visualised in cells expressing SseJ or a mutant SseJ (SseJ S151A). SseJ has homology to the GDSL-like lipolytic enzyme family [21] and shows deacylase, phospholipase and glycerophospholipid-cholesterol acyltransferase (GCAT) activity [22-24]. Ser151 in SseJ is the middle serine in a GDSL motif, which is present in 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 Δ sseJ *Salmonella*
153 (Fig. 3B). Typically, Δ 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 μ M

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

SseJ binds both RhoA and RhoC

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

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

Discussion

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

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

Materials and Methods

Reagents and Antibodies

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

Yeast Strains

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

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

***Salmonella* genomic library generation**

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

Constructs

SseJ was cloned from *S. Typhimurium* DNA by PCR. Primers were used to append a myc-tag to the SseJ PCR product along with 15bp regions of homology to the destination vector to allow for homologous recombination using In-Fusion cloning (Invitrogen). The myc-tagged SseJ DNA was inserted into the HindIII restriction enzyme site of the Δ pMEP4 vector [54] by homologous recombination. The S151A mutant was made by QuikChange site-directed mutagenesis (Stratagene) of the myc-SseJ construct as per the manufacturer's instructions.

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 50µl YPD
320 medium containing 40µM 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 H₂O 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% CO₂. Cells were
329 transfected with plasmid DNA using Fugene 6 (Roche Diagnostics) as per the
330 manufacturer's instructions. ΔpMEP4 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 µM CdCl₂ for 16-24h
334 before analyses.

J774.2 *Salmonella* infection.

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

Immunofluorescence

Cells were fixed with 4 % (w/v) formaldehyde in PBS for 20 min at 20°C. Cells to be immunolabelled for microtubules were rinsed with microtubule stabilising buffer (MTSB; 80mM PIPES, pH 6.8, 1mM MgCl₂, 4mM EGTA) then incubated in MTSB containing 0.05% (w/v) saponin (Sigma S-4521) for 1 min then fixed with 2% (w/v) formaldehyde, 0.05% (w/v) glutaldehyde in MTSB for 20 min. Cells immunolabelled for Glu-tubulin were fixed with -20°C MeOH for

5 min at -20°C. All fixed cells were incubated for 10 min in 50 mM NH₄Cl in PBS followed by 10 min in 0.2 % (w/v) BSA in PBS containing 0.5% (w/v) saponin (PBS-BS). Cells were immunolabelled with primary antibodies in PBS-BS for 1h at 20°C. Cells were rinsed 3 x 5 min with PBS-BS and then incubated with fluorescent secondary-antibodies in PBS-BS for 30 min at 20°C. Cells were rinsed 3 x 5 min with PBS-BS before being mounted in Mowiol. Fluorescence was imaged using a Zeiss LSM510 confocal microscope. All images are maximum intensity z-projections unless otherwise stated.

Cell lysates

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

Immunoprecipitation

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

Mass spectroscopy

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

RhoA activity assays

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

Live cell imaging

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

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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

Figure Legends

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

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

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

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

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

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

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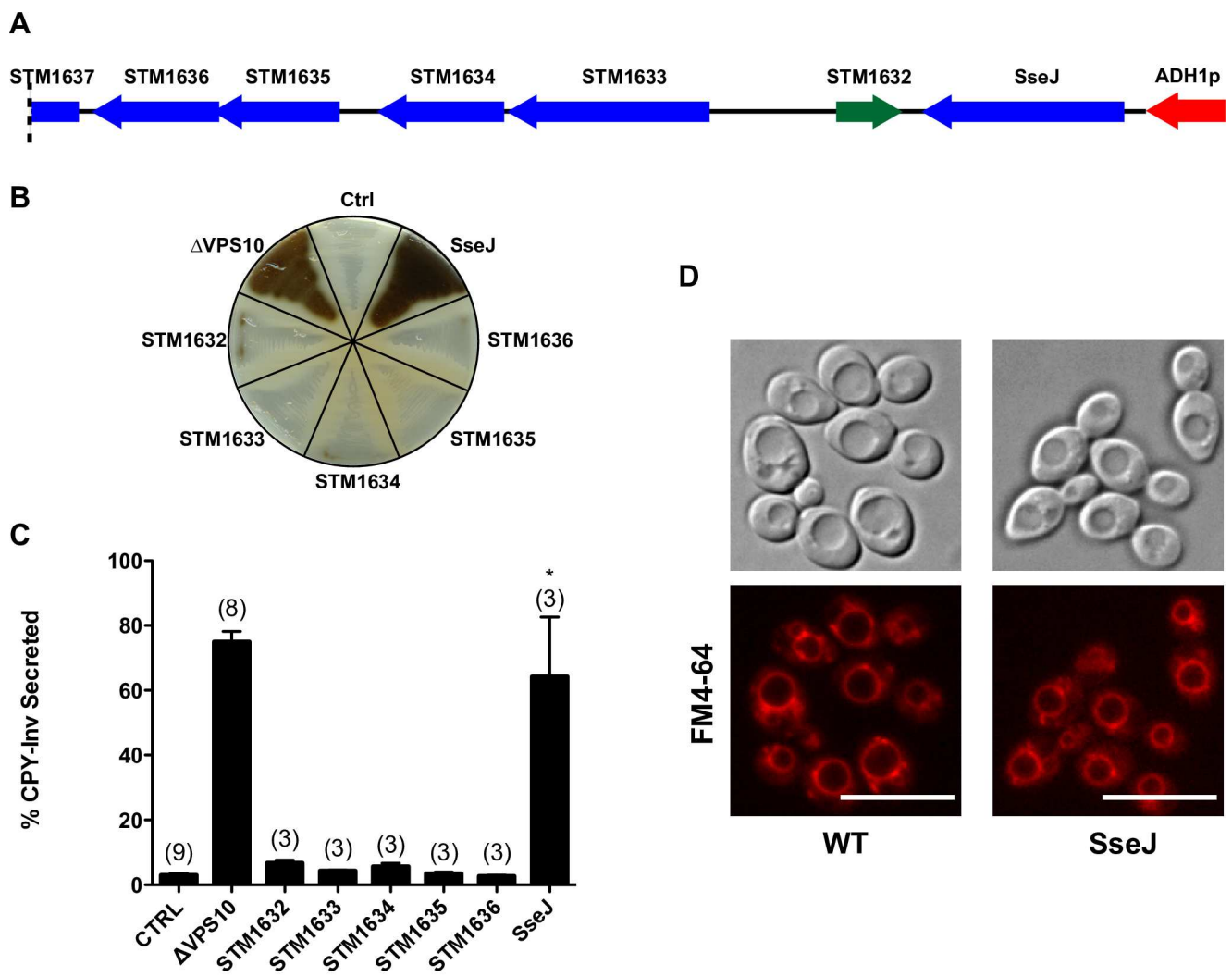


Fig 1

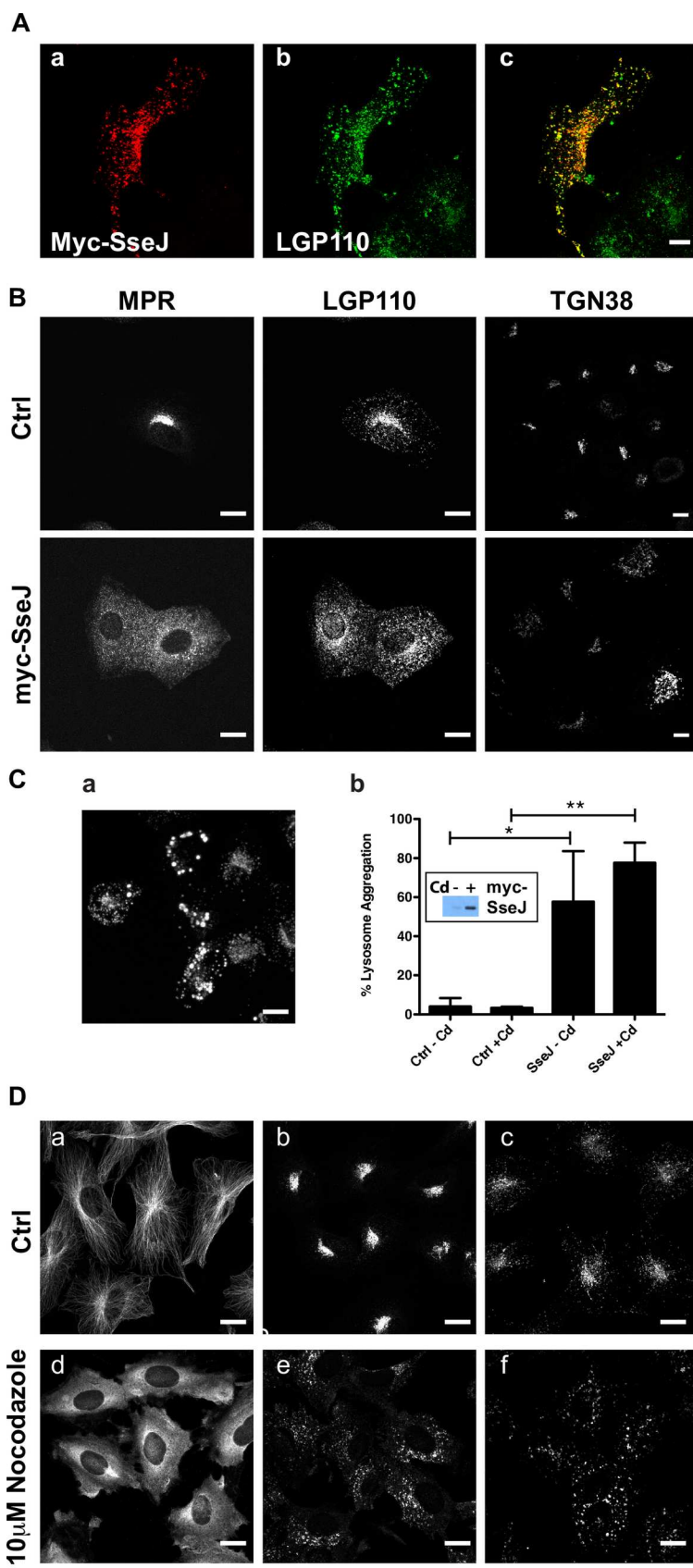


Fig 2

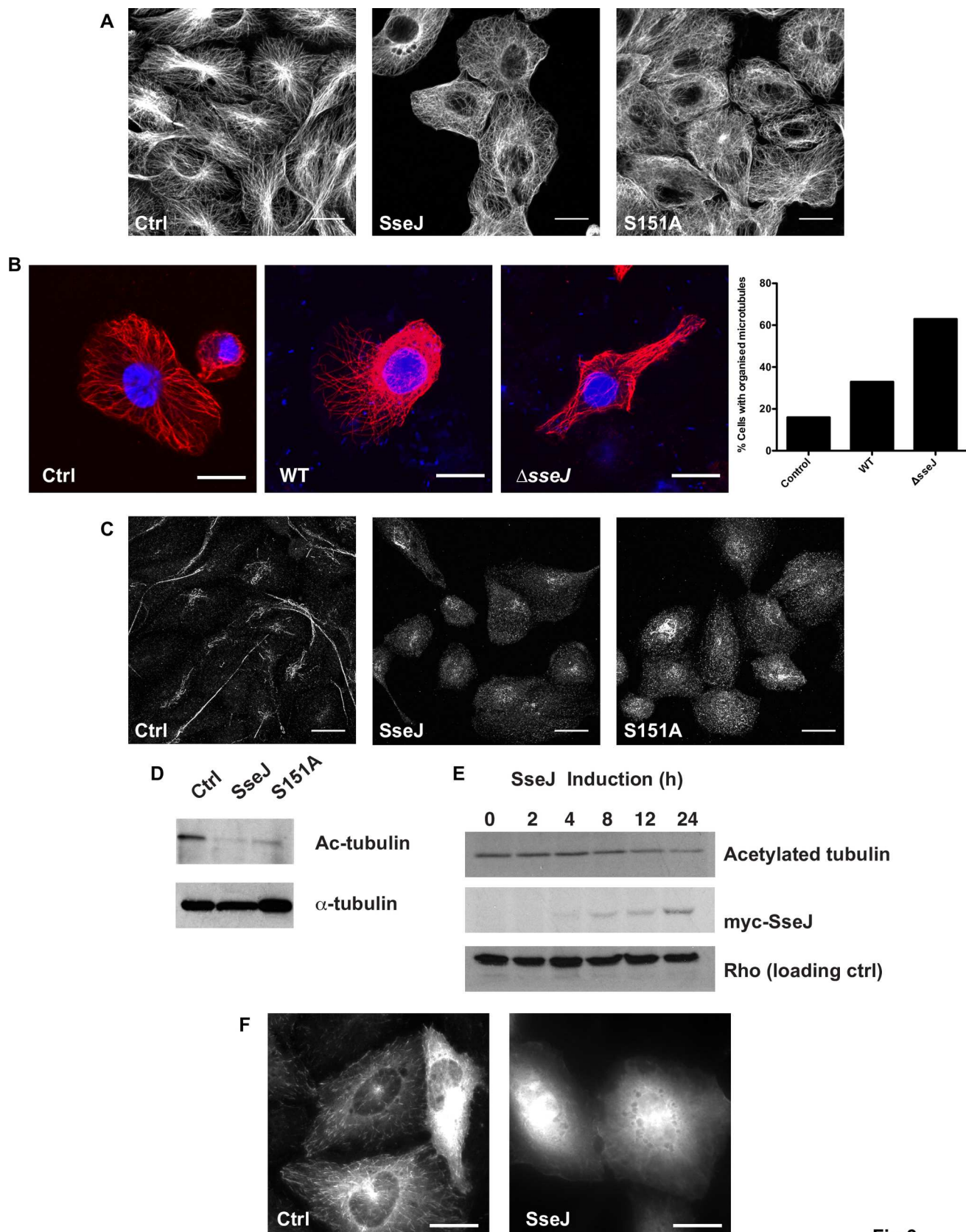
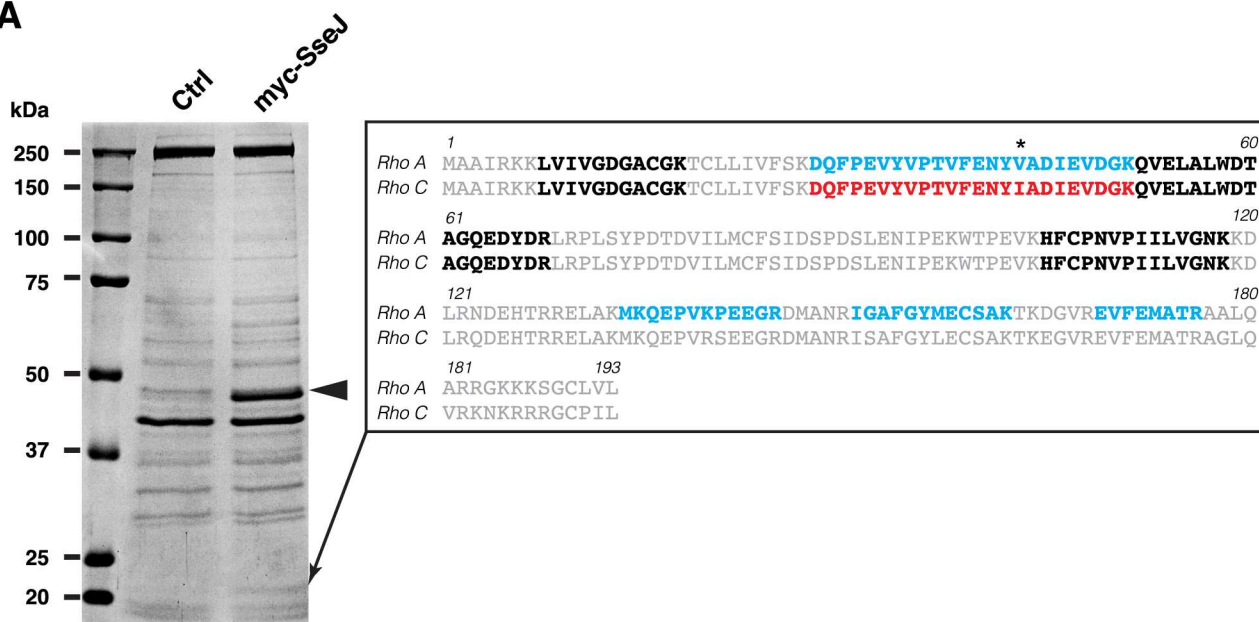
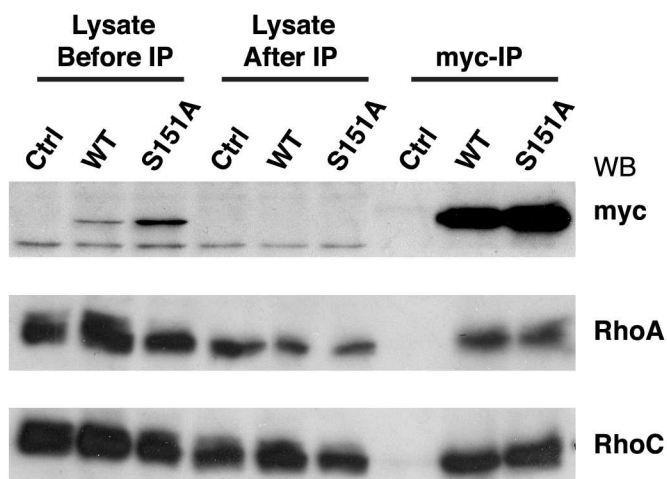


Fig 3

A



B



C

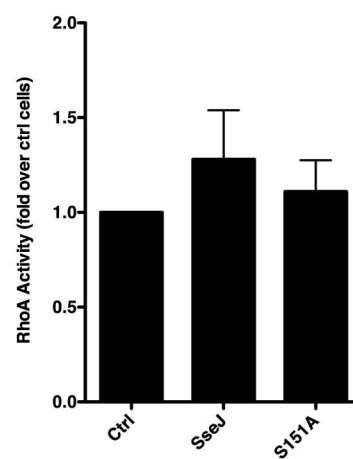


Fig 4