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## RESEARCH ARTICLE

# The *Salmonella* effector SifA initiates a kinesin-1 and kinesin-3 recruitment process mirroring that mediated by Arl8a and Arl8b

Ziyan Fang, Mathieu Fallet, Thomas Moest, Jean-Pierre Gorvel and Stéphane Méresse\*

## ABSTRACT

When intracellular, pathogenic *Salmonella* reside in a membrane compartment composed of interconnected vacuoles and tubules, the formation of which depends on the translocation of bacterial effectors into the host cell. Cytoskeletons and their molecular motors are prime targets for these effectors. In this study, we show that the microtubule molecular motor KIF1B $\beta$  (a splice variant of KIF1B), a member of the kinesin-3 family, is a key element for the establishment of the *Salmonella* replication niche as its absence is detrimental to the stability of bacterial vacuoles and the formation of associated tubules. Kinesin-3 interacts with the *Salmonella* effector SifA but also with SKIP (also known as PLEKHM2), a host protein complexed to SifA. The interaction with SifA is essential for the recruitment of kinesin-3 on *Salmonella* vacuoles whereas that with SKIP is incidental. In the non-infectious context, however, the interaction with SKIP is essential for the recruitment and activity of kinesin-3 only on a fraction of the lysosomes. Finally, our results show that, in infected cells, the presence of SifA establishes a kinesin-1 and kinesin-3 recruitment pathway that is analogous to and functions independently of that mediated by the Arl8a and Arl8b GTPases.

This article has an associated First Person interview with the first author of the paper.

**KEY WORDS:** *Salmonella*, Infection, Effector protein, Lysosome, Molecular motor, Membrane trafficking

## INTRODUCTION

Pathogenic bacteria that reside in host cells must adapt to this new environment. When bacteria are in a membrane compartment, the challenge is to shape it and to control its exchanges with the other compartments of the host cell. This is the task faced by *Salmonella*, a gram-negative intracellular pathogenic bacterium. Once inside a mammalian cell, a *Salmonella* bacterium resides in a membrane compartment, the *Salmonella*-containing vacuole (SCV), which matures along the endocytic pathway and eventually resembles the lysosomes of the host cell. Maturation of the SCV promotes the expression of a type III secretion system (T3SS) encoded by the second *Salmonella* pathogenicity island and named T3SS-2. This secretion system is used to translocate *Salmonella* effector proteins into the infected cell, allowing the bacteria to take control of certain host cell processes. The activities controlled by T3SS-2 effectors


include, among others: (1) manipulation of innate and adaptive immune responses; (2) remodelling of actin and microtubules cytoskeletons; and (3) regulation of SCV interaction with host compartments (for a review, see Jennings et al., 2017).

Very specific structures, known as *Salmonella*-induced filaments (SIFs), are formed in infected cells (Garcia-del Portillo et al., 1993). These are membrane tubules that emerge from the SCVs and stretch throughout the cell on the microtubule cytoskeleton (Rajashekar et al., 2008). SIFs have a similar composition to SCVs, that is, they are rich in lysosomal glycoproteins, such as LAMP1 and LAMP2. *Salmonella* also induce the formation of other types of tubules of different compositions, such as *Salmonella*-induced SCAMP3 tubules (SISTs) (Mota et al., 2009) and LAMP1-negative tubules (LNTs) (Schroeder et al., 2011). All these membrane tubules are grouped together under the generic term of *Salmonella*-induced tubules (SITs). SITs can be several tens of micrometres long and have an average diameter of 0.12 or 0.22  $\mu\text{m}$ , depending on whether they consist of a single or double membrane (Krieger et al., 2014). By promoting membrane exchanges, they contribute to the supply of nutrients to the bacteria and allow their multiplication (Liss et al., 2017). These structures do not form or are not detected in certain cells, such as macrophages, and it is not known why. It could be the lack of an essential host protein as suggested by the appearance of these structures after stimulation of macrophages (Knodler et al., 2003) or dendritic cells (Rajashekar et al., 2008).

The formation and membrane dynamics of these SITs are controlled by a number of T3SS-2 effectors. Among these, SifA is required for their formation (Stein et al., 1996). This effector recruits a cytosolic host protein called SKIP (also known as PLEKHM2) (Boucrot et al., 2005), which is a scaffold protein. In a non-infectious context, the interactions of SKIP with the small GTPases Arl8a and Arl8b (hereafter denoted Arl8a/b) (Rosa-Ferreira and Munro, 2011) and the HOPS complex (Sindhvani et al., 2017) favour its recruitment on lysosomes and homotypic membrane exchanges, respectively. SKIP also binds kinesin-1 family members, molecular motors that move to the plus end of microtubules (Boucrot et al., 2005). Kinesin-1 is also recruited on the surface of *Salmonella* compartments by its interaction with PipB2 (Henry et al., 2006). In the absence of this effector, SITs are shorter, which is likely related to a function of tubule elongation by the kinesin-1 (Knodler and Steele-Mortimer, 2005). The respective roles of SifA and PipB2 in the recruitment and activation of kinesin-1 are not yet well understood. A recent study shows that, *in vitro*, the interaction with PipB2 activates the molecular motor (Alberdi et al., 2020). However, this is probably not the case in infected cells since the phenotypes observed with different *Salmonella* mutants suggest that the SifA–SKIP complex is required for the activation of kinesin-1 (Dumont et al., 2010; Schroeder et al., 2011) and the interaction with SKIP has been shown to mediate kinesin-1 activation (Sanger et al., 2017). In the absence of SifA or SKIP, and thus of SITs, significant accumulations of membrane-bound

Aix Marseille University, CNRS, INSERM, CIML, Marseille, France.

\*Author for correspondence (meresse@ciml.univ-mrs.fr)

 Z.F., 0000-0001-7193-9398; M.F., 0000-0001-8144-6159; T.M., 0000-0002-1240-3953; J.-P.G., 0000-0002-2829-9804; S.M., 0000-0001-6578-5177

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T3SS-2 effectors and also of PipB2-dependent kinesin-1 are observed on SCVs (Boucrot et al., 2005; Henry et al., 2006; Schroeder et al., 2011), and this can be explained by the strong contraction of the membrane surface of the bacterial compartment. With a *sopD2* mutant, a reduction in the number and length of SITs and discontinuous labelling of LAMP1 was observed (Jiang et al., 2004). These altered structures were also observed in the absence of *sseF* or *sseG*, and have since been referred to as pseudo-SIFs (Kuhle and Hensel, 2002). It should be noted that SopD2 binds and inhibits the nucleotide exchange activity of Rab7, thus limiting the recruitment of the host effector RILP (a Rab-interacting lysosomal protein) and consequently of dynein, a molecular motor that moves centripetally on microtubules (D'Costa et al., 2015). Finally, the lipid composition of SCVs is another determinant factor in the formation of SITs. SseJ, which has enzymatic activity to esterify cholesterol (Nawabi et al., 2008; Ohlson et al., 2005) and binds to oxysterol binding protein 1 (OSBP1) (Kolodziejek et al., 2019), enhances the ability of ectopically expressed SifA to induce membrane tubules in HeLa cells (Ohlson et al., 2008).

It therefore appears that the formation of tubules involves the activities of several effectors involved in the recruitment and activity of molecular motors. While dynein is necessary for centripetal transport of SCVs (Harrison et al., 2004) and then is excluded from SCVs and SITs (Marsman et al., 2004), the role of kinesins seems to persist throughout the infection cycle. In a siRNA screening carried out in HeLa cells, Kaniuk et al. showed that SIF formation involves several kinesins other than kinesin-1 (Kaniuk et al., 2011). Various kinesins have been implicated in the intracellular positioning of lysosomes, in particular kinesin-1 (Tanaka et al., 1998), kinesin-2 (Brown et al., 2005), kinesin-3 (Matsushita et al., 2004) and kinesin-13 (Santama et al., 1998) family members. A recent study by Guardia et al. (Guardia et al., 2016) showed that, in HeLa cells, it is kinesin-1 and kinesin-3 whose expression inhibition has the most impact on the distribution of lysosomes.

In this study, we identified kinesin-3, specifically KIF1B $\beta$  (a splice variant of KIF1B), as an essential molecular motor in *Salmonella* infections playing an important role in the stability of bacterial vacuoles and the formation of emerging tubules. Overall, we show a striking parallel in the interactions involved in the recruitment of kinesin-1 and kinesin-3 by the Arl8a/b GTPases and the effector SifA in infected cells.

## RESULTS

### Kinesin-3 is present on SCVs and SITs and its recruitment depends on T3SS-2

KIF1A and KIF1B $\beta$  are homologous members of the kinesin-3 family with a similar domain organization, and both are involved in the transport of synaptic vesicle precursors (Zhao et al., 2001) and lysosomes (Guardia et al., 2016). Given the strong similarity between the membranes of lysosomes and those of *Salmonella* compartments, it appeared relevant to analyse the presence of these molecular motors on SCVs and SITs. For this, HeLa cells were transfected with plasmids for the expression of tagged human KIF1A or KIF1B $\beta$ , infected with *Salmonella enterica* serovar Typhimurium for 16 h and observed by confocal microscopy. We found the presence of KIF1A (Fig. S1A) and KIF1B $\beta$  (Fig. 1A) on SCVs and SITs, as identified by their LAMP1 labelling. In the further course of this study we used KIF1B $\beta$  (unless otherwise specified) which, unlike KIF1A, is ubiquitously expressed. KIF1B $\beta$  is hereafter referred to as kinesin-3.

We compared the recruitment on SCVs of kinesin-3 and kinesin-1, the latter resulting from the co-expression of HA-tagged rat

KIF5C and mouse KLC2. As kinesin-1 accumulates on SCVs in the absence of SifA (Boucrot et al., 2005; Zhao et al., 2015), we tested in addition to wild-type *Salmonella*, a  $\Delta$ *sifA* mutant strain and, as a control, a  $\Delta$ *sseF* mutant. Kinesin-1 could not be detected on either wild-type SCVs or those containing the  $\Delta$ *sseF* mutant. However, about half of these SCVs ( $56\pm 16\%$  and  $47\pm 11\%$  for wild-type and  $\Delta$ *sseF*, respectively; mean $\pm$ s.d.) were positive for kinesin-3 (Fig. 1B, C and Fig. S2). We detected kinesin-1 in a large proportion of the  $\Delta$ *sifA* SCVs ( $75\pm 6\%$ ) which, however, were rarely positive for kinesin-3 ( $12\pm 3\%$ , Fig. 1B,C). These results indicate that kinesin-3 is, like kinesin-1, present on *Salmonella* compartments, but that the mechanisms regulating the recruitment of these molecular motors differ.

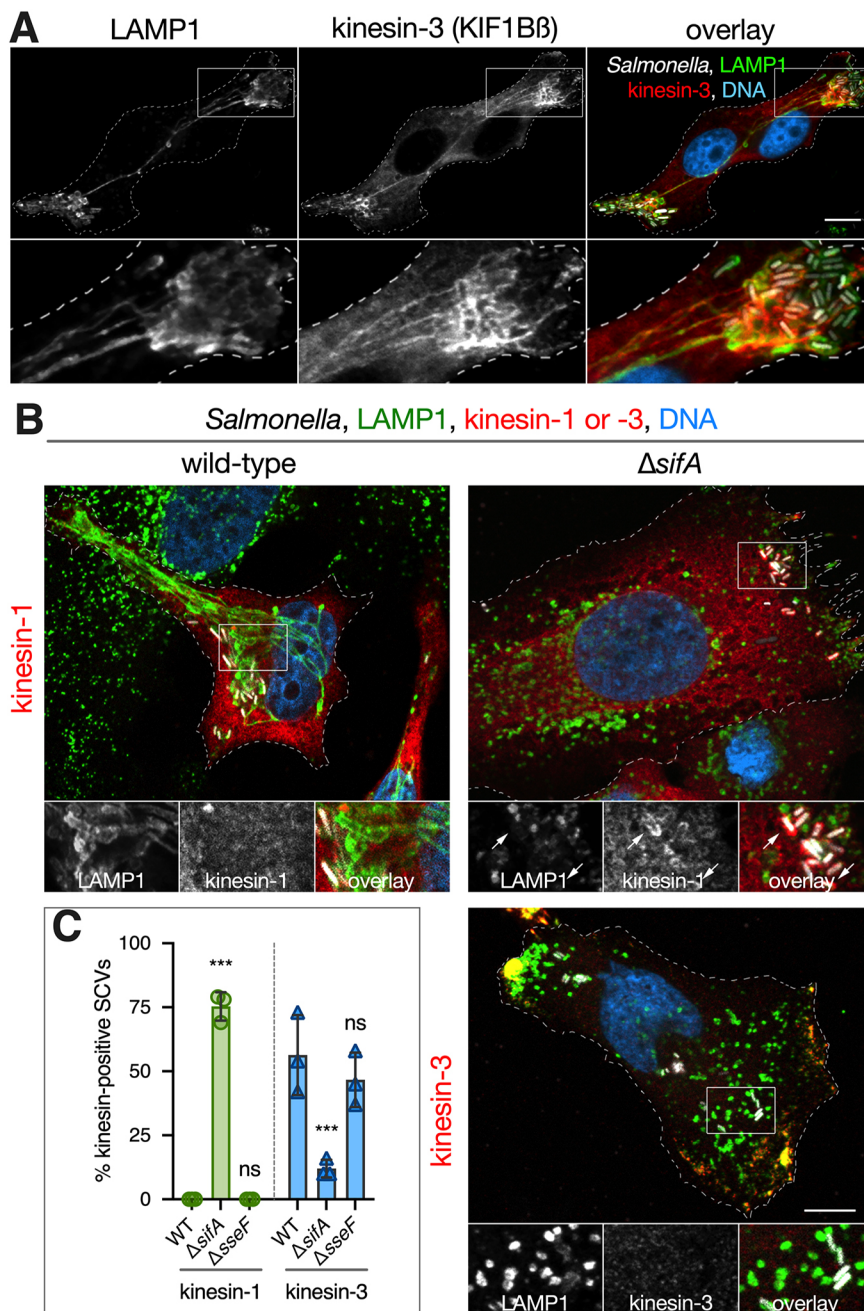
### Consequence of kinesin-1 and kinesin-3 silencing in *Salmonella*-infected cells

To assess the importance of these kinesins on the positioning and membrane dynamics of SCVs and SITs, and on the ability of *Salmonella* to replicate within the vacuoles, we inhibited their expression. We treated HeLa cells with siRNA pools targeting the kinesin-1 (KIF5B) or kinesin-3 (KIF1B) (Guardia et al., 2016). A siRNA that inhibited SKIP expression (Boucrot et al., 2005) and a non-targeting siRNA pool served as a positive and negative control, respectively.

Prior to their use in the context of infection, we verified that siRNAs pools targeting kinesin-1 or kinesin-3 inhibited the expression of molecular motors (Fig. S3A). Note that, compared to the control, we also observed a slight decrease ( $\sim 10\%$ ) in the level of kinesin-3 in cells treated with the siRNA pool targeting kinesin-1. We also monitored their effects on the positioning of the LAMP1 compartment. Although present in other endocytosis compartments, LAMP1 is an established marker of lysosomes and in the remainder of this paper LAMP1-positive vesicles will be referred to as lysosomes. Silencing of either kinesin-1 or kinesin-3 resulted in the concentration of lysosomes in the juxtannuclear region (Fig. S3B) and a near absence of lysosomes at the cell periphery was observed in the absence of both kinesins, a phenotype very similar to that observed in the absence of SKIP.

These cells were used to analyse the role of kinesins in SCV positioning, SIT formation and bacterial replication. HeLa cells were infected with wild-type *Salmonella* prior to immunostaining for LAMP1. Regarding the positioning of the SCVs at 16 h post infection (hpi), we analysed their distribution with respect to the nucleus and the cell border from confocal images using the ImageJ Radial Profile plugin. We calculated the fractional distance (FD) (Guardia et al., 2016), defined as the ratio of the mean distance between objects (here the SCVs) and the edge of the nucleus to the difference between the mean radius of the cell and the mean radius of the nucleus. FD tends towards 0 or 1 depending on whether the objects are at the edge of the nucleus or the cell (Fig. S4). Not surprisingly, given our previous results (Boucrot et al., 2005), we found that there was a more peripheral positioning of the SCVs in the absence of SKIP (FD of  $0.44\pm 0.03$  versus  $0.23\pm 0.02$  for the control; mean $\pm$ s.d.) (Fig. 2A,B). Inhibition of kinesin-3 expression positioned the bacterial vacuoles slightly closer to the nuclei (FD= $0.18\pm 0.01$ ), whereas inhibition of kinesin-1 showed no effect (FD= $0.27\pm 0.01$ ). However, we observed that in the absence of kinesin-1 the intracellular colonies were more compact (Fig. 2A), confirming a previous observation made in kinesin-1 knockout mouse macrophages (Alberdi et al., 2020). These FD values confirmed the initial observations and scoring from epifluorescence microscopy experiments (Fig. S3C).





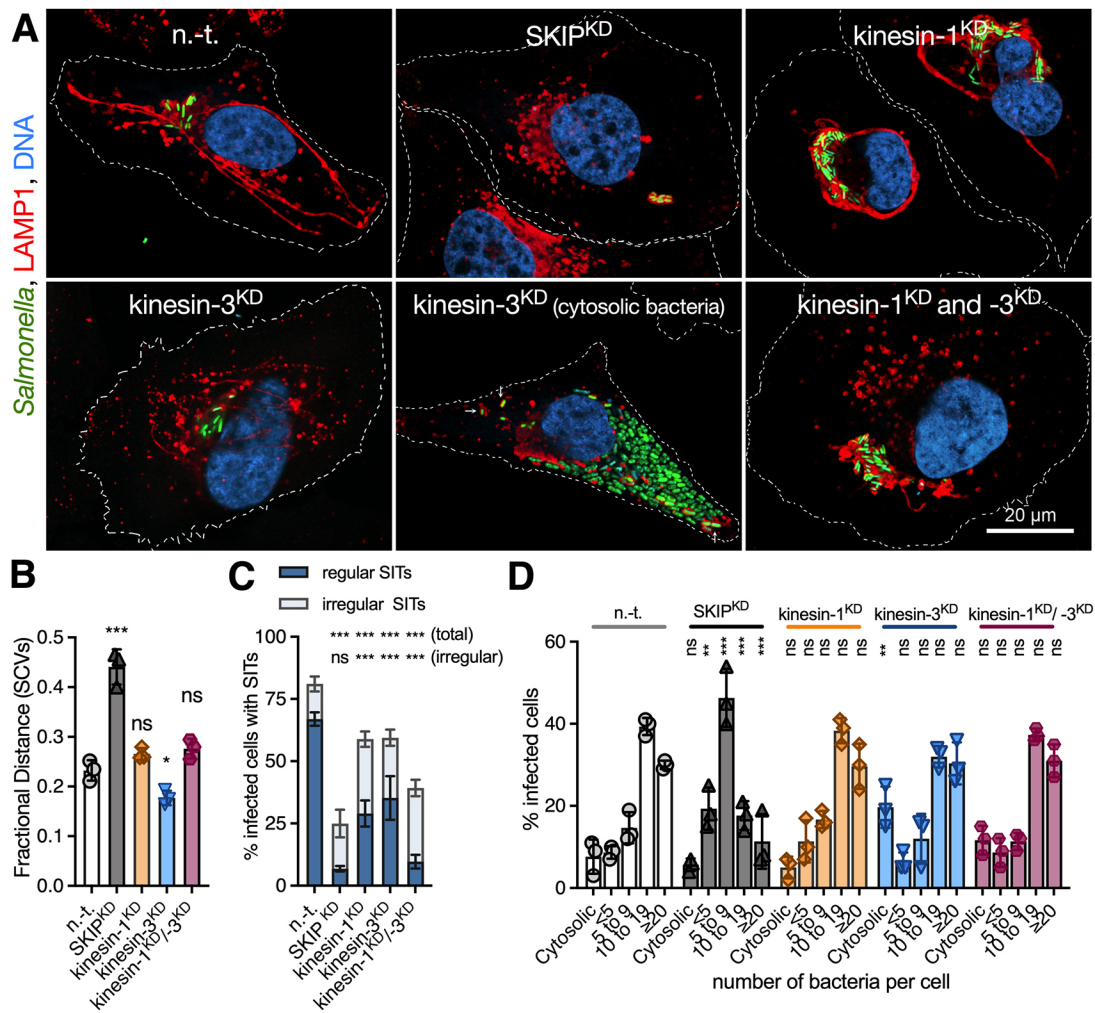
**Fig. 1. Kinesin-3 is present on *Salmonella* compartments.** HeLa cells were transfected with plasmids for the expression of kinesin-1 (HA-KLC2 and HA-KIF5C) or kinesin-3 (FLAG-KIF1B $\beta$ ) and further infected with various *Salmonella* strains expressing GFP. After 16 h of infection, cells were fixed, immunostained and imaged for GFP (white), LAMP1 (green), kinesin-1 or -3 (red) and DNA (blue) using confocal microscopy. WT, wild type. (A) In cells infected with wild-type *Salmonella*, kinesin-3 is present on SCVs and SITs. The bottom row shows the insets enlarged three times. Scale bars: 10  $\mu$ m (main images; represents 3.3  $\mu$ m on the magnified views). (B) Unlike kinesin-3, kinesin-1 is not detected on wild-type *Salmonella* compartments (upper left images). In contrast, kinesin-1 is detected on  $\Delta sifA$  vacuoles whereas kinesin-3 is not (images on the right, top and bottom, respectively). Enlarged images show single labelling for LAMP1 or kinesins. Arrows point to kinesin-1-positive SCVs. Scale bars: 10  $\mu$ m (main images; represents 5  $\mu$ m on the magnified views). Dashed lines in A and B show edges of cells. (C) The percentage of kinesin-positive bacteria was scored for wild-type *Salmonella* and  $\Delta sifA$  and  $\Delta sseF$  mutants. Data are means  $\pm$  s.d. of three independent experiments. The scoring was performed on more than 50 infected and transfected cells per experiment. Not significant (ns),  $P > 0.05$ ; \*\*\* $P < 0.001$  (two-way ANOVA and Dunnett's multiple comparisons test to compare the results obtained for the mutant strains with those of wild-type *Salmonella*).

SITs normally emerge from the SCVs and stretch in all directions to reach the cell periphery (Fig. 2A). In control cells, these tubules were observed in a large majority of infected cells ( $\sim 80\%$ ) whereas in the absence of SKIP, which is essential for their formation (Boucrot et al., 2005), these structures were rarely seen (Fig. 2A,C). We noted a moderate reduction in the percentage of infected cells with SITs in the absence of either of the kinesins. However, about half of these structures were morphologically altered. These SITs were short, thick and frequently wrapped around the nucleus in the absence of kinesin-1. They were most often short and abnormally thin in the absence of kinesin-3 (Fig. 2A). These are hereafter referred to as irregular SITs. Concomitant inhibition of the expression of both kinesins markedly reduced the occurrence of SITs, which were also predominantly irregular and rather similar to those observed in cells not expressing kinesin-3. These results

indicate that kinesin-3 is involved in the formation of the thick SITs observed in the absence of kinesin-1 and that the latter is necessary for their elongation towards the cell periphery.

We then analysed the impact of kinesins on *Salmonella* intracellular replication at 12 hpi. The intracellular replication is a result of the capacity of SVCs to support bacterial growth and of their stability. Under certain circumstances, for example, in the absence of SifA, SCVs are unstable, resulting in the release of bacteria into the cytoplasm and this has, depending on the infected cell, very different consequences (Beuzón et al., 2000). In phagocytic cells whose cytoplasm is bactericidal for *Salmonella*, this leads to a lower net replication for this mutant than for a wild-type strain (Beuzón et al., 2002). Conversely, a higher net replication of this mutant is observed in epithelial cells in which cytoplasmic replication is much faster than in SCVs, a phenomenon





**Fig. 2. Consequences of kinesin-1 and/or kinesin-3 knockdown on the positioning and membrane dynamics of *Salmonella* compartments and on intravacuolar replication.** HeLa cells were transfected with siRNA pools for kinesin-1 and/or kinesin-3 knockdown. A non-targeting siRNA (n.-t.) pool and a siRNA against SKIP were used as negative and positive controls, respectively. Transfected cells were infected with wild-type (WT) *Salmonella* for 12 (D) or 16 (A–C) hours and immunostained. (A) Confocal microscopy images representative of the phenotypes observed. The arrows in the image illustrating the presence of bacteria in the cytosol point to bacteria still present in SCVs. Dashed lines show edges of cells. (B) Random confocal images were used to calculate the mean FD for SCVs under the different cell conditions. (C) Percentages of infected cells with regular or irregular (short, thick or thin) SITs. (D) Percentages of cells infected with cytosolic bacteria and scoring of the number of intravacuolar bacteria. Cytosolic bacteria were identified by the absence of vacuole (LAMP1 labelling). The cells with intravacuolar bacteria were categorized according to the number of bacteria contained. For B–D, at least 50 infected cells per experiment were analysed. Data are means±s.d. of at least three independent experiments. Not significant (ns),  $P>0.05$ ; \*,  $P<0.05$ ; \*\*,  $P<0.01$ ; \*\*\*,  $P<0.001$  [Ordinary one-way ANOVA and Dunnett's multiple comparisons test (B), two-way ANOVA and Tukey's multiple comparisons test (C), and two-way ANOVA and Dunnett's multiple comparisons test (D) were used to compare the results in the knocked-down and control (n.-t.) cells].

called hyper-replication (Knodler et al., 2014). The measure of colony-forming unit in HeLa cells does not allow the dissociation between vacuolar versus cytoplasmic replication. We therefore scored by microscopic observation, on the one hand, the cells with cytoplasmic bacteria and, on the other hand, the cells with a vacuolar localization of *Salmonella*. In the absence of kinesin-3, we found a clear increase in the number of cells with cytoplasmic hyper-replication ( $7.7\pm 4.2\%$  and  $19.7\pm 5\%$  in the presence and absence of kinesin-3, respectively; mean±s.d.), reflecting an enhanced instability of SCVs. We did not find such instability in cells that did not express kinesin-1 or those expressing neither kinesin-1 nor kinesin-3 (Fig. 2A,D). The cells with intravacuolar bacteria were classified into four categories: cells containing 1 to 4, 5 to 9, 10 to 19, and 20 or more bacteria. In the group of cells transfected with a non-targeting siRNA, the majority of cells (~70%) contained more than 10 bacteria, roughly equally divided between the 10–19

and 20+ categories. In cells not expressing SKIP, and as expected (Boucrot et al., 2005), we observed a marked decrease in intracellular replication, with the category of cells containing between 5 and 9 bacteria accounting for half of the total. In cells not expressing kinesin-1 or kinesin-3 or none of the kinesins, we observed a distribution in the categories very similar to that of the control group. Thus, it appears that, unlike SKIP, kinesin-1 and -3 are not required for intravacuolar replication in HeLa cells. However, kinesin-3 was found to be an essential factor for the stability of SCVs.

#### SifA is required for the recruitment of kinesin-3 on SCVs

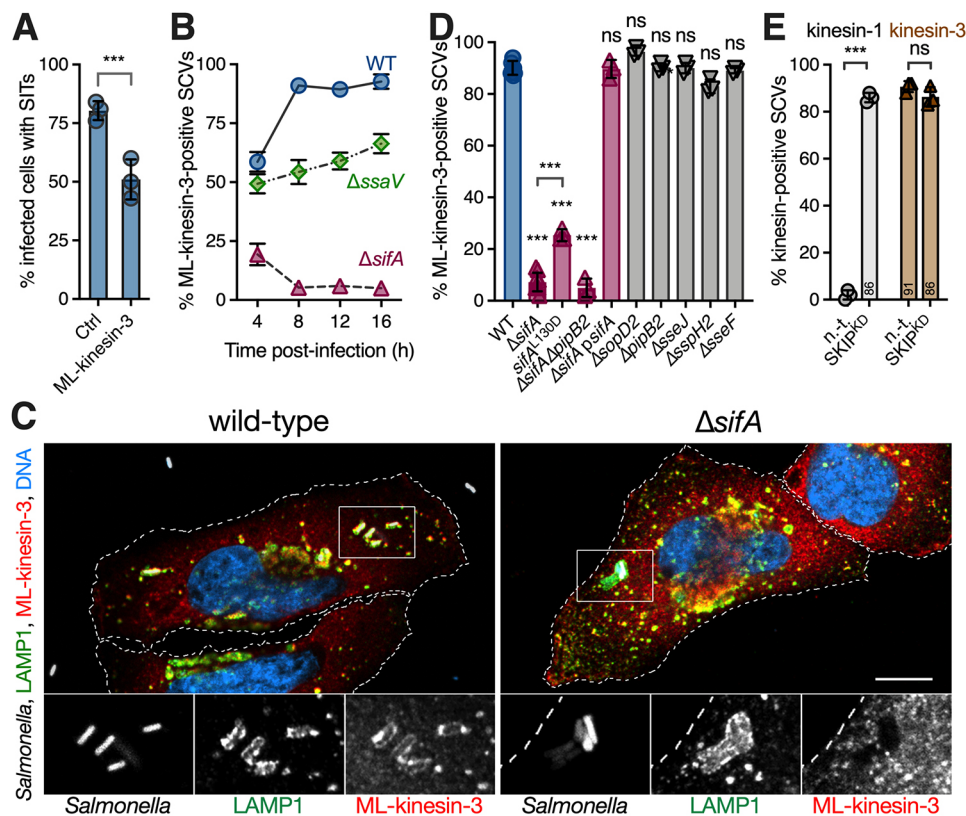
Having shown the importance of kinesin-3 in the positioning and membrane dynamics of SCVs and SITs, it was then important to dissect its recruitment mechanism. The cells were infected with *Salmonella* strains expressing a 2HA-tagged T3SS-2 effector

(PipB2 or SseJ) in order to obtain unambiguous labelling of the *Salmonella* compartments and thus overcome the difficulties caused by the loss of LAMP1 labelling characteristic of the  $\Delta sifA$  mutant (Beuzón et al., 2000). In addition, we used a motor-less form of kinesin-3 [ML-kinesin-3, human KIF1B $\beta$  (363-stop); Matsushita et al., 2004] whose overexpression has a dominant-negative effect on the formation of SITs (Fig. 3A) but marks the SCVs well, facilitating their counting. With this system, we followed the evolution over time of the presence of kinesin-3 on SCVs enclosing wild-type bacteria, and mutant  $\Delta sifA$  or  $\Delta ssaV$  bacteria. For the latter strain, which expresses a non-functional T3SS-2 and is therefore defective in the secretion of all corresponding effectors, counting was performed using LAMP1 labelling of *Salmonella* vacuoles. At 4 hpi, the fractions of vacuoles positive for ML-kinesin-3 were not substantially different for the wild-type and  $\Delta ssaV$  strains (~50%) but were lower for the  $\Delta sifA$  mutant (~20%) (Fig. 3B). The fraction of positive  $\Delta ssaV$  SCVs then increases slightly and reaches 66% at 16 hpi. For the wild-type strain, the percentage increases sharply at 8 hpi and stabilizes at ~90%, while at the same time it

decreases and stabilizes at ~5% for the  $\Delta sifA$  strain. Confocal images illustrate that at 8 hpi, even though LAMP1 is still very much present, ML-kinesin-3 is not detected on  $\Delta sifA$  SCVs (Fig. 3C).

We then tested several other bacterial strains at 16 hpi to verify the specificity of the impact of SifA on kinesin-3 recruitment. The results (Fig. 3D) show that the presence of a plasmid allowing the expression of SifA under the control of its own promoter ( $\Delta sifA$  *psifA* strain) complements the kinesin-3 recruitment defect. They also indicate that neither PipB2, which interacts with kinesin-1, nor the other effectors tested, which we selected for their involvement in SIT formation, are required for kinesin-3 recruitment. Finally, we tested the possibility of kinesin-3 exclusion due to the accumulation of kinesin-1 on the  $\Delta sifA$  SCV and, for this, used a  $\Delta sifA$   $\Delta pipB2$  strain (Henry et al., 2006). In cells infected with this strain, the SCVs were as negative for kinesin-3 as with the  $\Delta sifA$  SCVs, thus invalidating that hypothesis.

Altogether, these results indicate that the expression of T3SS-2, which occurs between 4 and 8 hpi, and the associated translocation of SifA are necessary for the recruitment of kinesin-3 on *Salmonella* compartments.



**Fig. 3. SifA is necessary for the recruitment of kinesin-3 on SCVs.** (A) A motor-less (ML) form of kinesin-3 has a dominant-negative activity on the formation of SITs. HeLa cells expressing or not ML-kinesin-3 were infected with wild-type (WT) *Salmonella*, fixed at 16 hpi and immunostained for LAMP1. Infected cells were scored for the presence of SITs. (B) Percentages of ML-kinesin-3 positive SCVs at different times of infection for wild-type *Salmonella* and mutants  $\Delta ssaV$  and  $\Delta sifA$ . The cells were fixed and immunostained for 2HA-PipB2 (WT and  $\Delta sifA$ ) or LAMP1 ( $\Delta ssaV$ ) and ML-kinesin-3. The percentages of SCVs (HA or LAMP1-positive bacteria) positive for ML-kinesin-3 were determined by epi-fluorescence microscopy. (C) Images illustrating the labelling for ML-kinesin-3 on LAMP1-positive SCVs at 8 hpi. Wild-type *Salmonella* and a  $\Delta sifA$  mutant are enclosed in ML-kinesin-3-positive or -negative vacuoles, respectively. Enlarged images showing single labellings are presented below. Dashed lines show edges of cells. Scale bars: 10  $\mu$ m (main images; represents 5  $\mu$ m on the magnified views). (D) The presence of ML-kinesin-3 on SCVs in cells infected with various *Salmonella* strains was analysed. The cells were fixed at 16 hpi, and immunostained and scored by epi-fluorescence microscopy. (E) SKIP is not essential for the recruitment of kinesin-3 on SCVs. Control HeLa cells (n.t.) or cells not expressing SKIP (SKIP<sup>KD</sup>), were then transfected with plasmids for the expression of kinesin-1 or ML-kinesin-3 and infected with wild-type *Salmonella* for 16 h. Fixed cells were immunostained for LAMP1 and kinesin-1 or ML-kinesin-3. SCVs positive for either kinesin were counted as described above. Data in A, B, D and E are means  $\pm$  s.d. of at least three independent experiments. Not significant (ns),  $P > 0.05$ ; \*\*\* $P < 0.001$  [two-way ANOVA and Šidák multiple comparisons test (A,E); ordinary one-way ANOVA and Dunnett's multiple comparisons tests were used to compare the results obtained for the mutant strains with those of wild-type *Salmonella* (D); unpaired two-tailed *t*-test was used to compare  $\Delta sifA$  and *sifA*<sup>L300D</sup> strains].

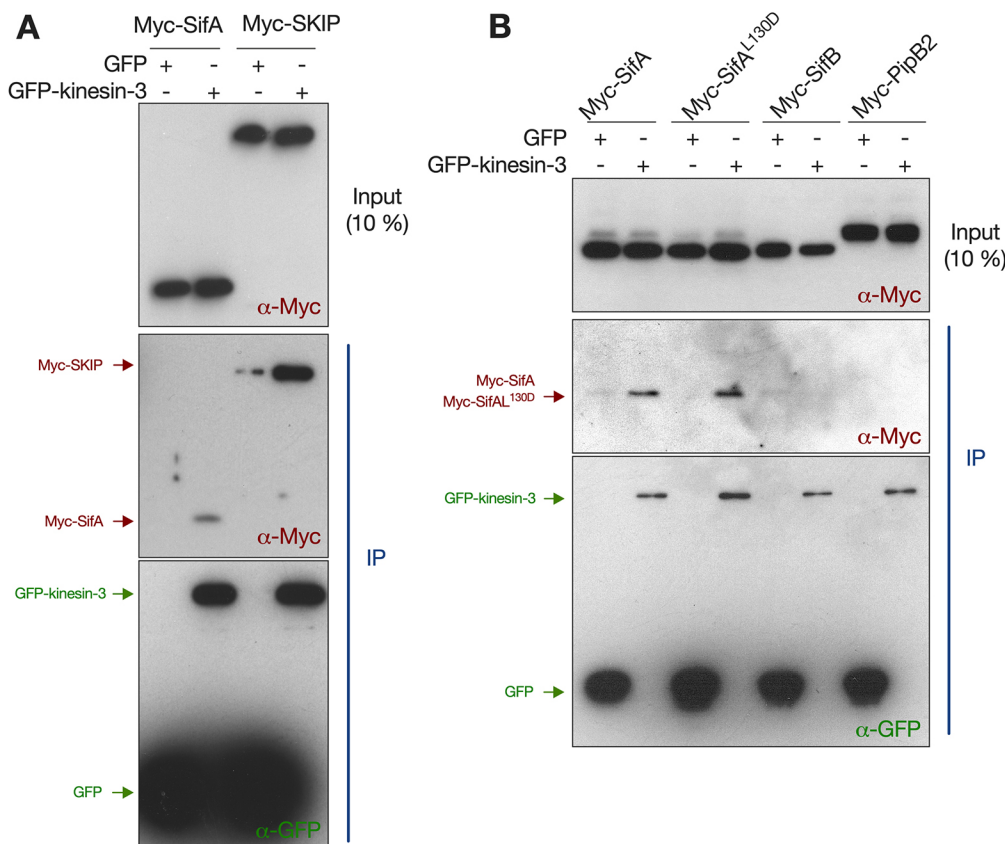
### Kinesin-3 interacts with both SifA and SKIP

After showing the role of SifA in the recruitment of kinesin-3, we then proceeded to perform experiments to understand the interactions involved. We tested the possibility of an interaction with SifA or, as in the case of kinesin-1, an interaction with SKIP. For this purpose, cells were transfected with plasmids for the expression of GFP or GFP–kinesin-3 as well as different Myc-tagged T3SS-2 effectors or host proteins. The cell lysates were subjected to immunoprecipitation of GFP, and the bead-bound proteins were analysed by western blotting. This allowed us to see that both SifA and SKIP co-immunoprecipitated with GFP–kinesin-3 but not with GFP (Fig. 4A). Likewise, we found interactions between SifA, SKIP and the neuronal protein KIF1A (Fig. S1B). In order to assess the quality of these interactions, we tested other T3SS-2 effector proteins. We did not find any interaction of kinesin-3 with SifB, which has a strong sequence homology with SifA (Miao and Miller, 2000), nor with PipB2, which binds the light chains of kinesin-1 (Henry et al., 2006) (Fig. 4B). We tested which of the N- or C-terminal domains of SifA and SKIP are involved in interactions with kinesin-3. The results suggest multiple interactions with SKIP as both its N-terminal and its C-terminal portions coprecipitate with the molecular motor (Fig. S5A). For SifA, we found that the N-terminal domain, which also binds SKIP, is responsible for the interaction (Fig. S5B). Co-immunoprecipitation was detected with the mutant SifA<sup>L130D</sup>, which does not bind SKIP (Diacovich et al., 2009; Ohlson et al., 2008), ruling out a sole indirect interaction via this protein (Fig. 4B). We tested the interactions in cells expressing both SifA or SifA<sup>L130D</sup> and SKIP. The results (Fig. S6A) did not indicate competition or cooperation for interaction with kinesin-3. Finally, we tested the interaction between kinesin-3 and PLEKHM1. This host protein has a domain

organization similar to that of SKIP (also called PLEKHM2) and also interacts directly with SifA (McEwan et al., 2015) but we did not detect any interaction with kinesin-3 (Fig. S6B).

In order to better appreciate the relative importance of interactions involving SifA and SKIP for the recruitment of kinesin-3 on *Salmonella* compartments, we analysed, by fluorescence microscopy, the recruitment of the molecular motor on SCVs enclosing a  $\Delta sifA$  mutant expressing SifA<sup>L130D</sup> from a plasmid (Fig. 3D). We found a lower percentage of ML-kinesin-3-positive vacuoles for this strain ( $25\pm 3\%$ ; mean $\pm$ s.d.) than found for the wild-type bacteria ( $91\pm 3\%$ ), but also significantly higher than found for the  $\Delta sifA$  mutant ( $7.5\pm 4\%$ ). This result confirms that SifA<sup>L130D</sup> is alone capable of recruiting kinesin-3 but less efficiently than the regular protein. Since this mutant carries a mutation in the N-terminal domain that interacts with kinesin-3, and although we have biochemically demonstrated an interaction between these two proteins, an impact of the mutation on this result could not be excluded. We therefore used HeLa cells in which SKIP expression was inhibited (Boucrot et al., 2005). These and other control cells were then infected with wild-type *Salmonella*. As a control for SKIP inhibition, we examined the recruitment of kinesin-1 given that this motor is recruited on wild-type SCVs in the absence of SKIP (Boucrot et al., 2005). We observed in the absence of SKIP a recruitment of kinesin-1 on  $86\pm 2\%$  of wild-type SCVs against  $2\pm 2\%$  in control cells, thus confirming the efficient elimination of SKIP expression, and kinesin-3 recruitment in a very high fraction of SCVs independently of the presence of SKIP ( $91\pm 2.3\%$  and  $86\pm 4.2\%$  in the presence and absence of SKIP, respectively) (Fig. 3E).

Taken together, these data indicate that kinesin-3 interacts with SifA and SKIP but that the presence of SKIP is not



**Fig. 4. Co-immunoprecipitation tests show interactions of kinesin-3 with SifA and SKIP.** Cos-7 (A) or HeLa (B) cells were transfected with plasmids for the expression of GFP or GFP–kinesin-3 and various Myc-tagged proteins. Immunoprecipitations were performed with GFP-Trap beads. Input and immunoprecipitated proteins (IP) were analysed by western blotting using anti-Myc and anti-GFP antibodies. (A) Kinesin-3 interacts specifically with SifA and SKIP. Both proteins co-immunoprecipitate with GFP-kinesin-3 but not with GFP. (B) The interaction of kinesin-3 with SifA is independent of SKIP. SifA and a SifA<sup>L130D</sup> mutant that does not interact with SKIP co-immunoprecipitate specifically with GFP-kinesin-3. This is not the case for the T3SS-2 effectors SifB and PipB2. Blots shown are representative of at least three experiments.



essential for the recruitment of the motor on *Salmonella* compartments.

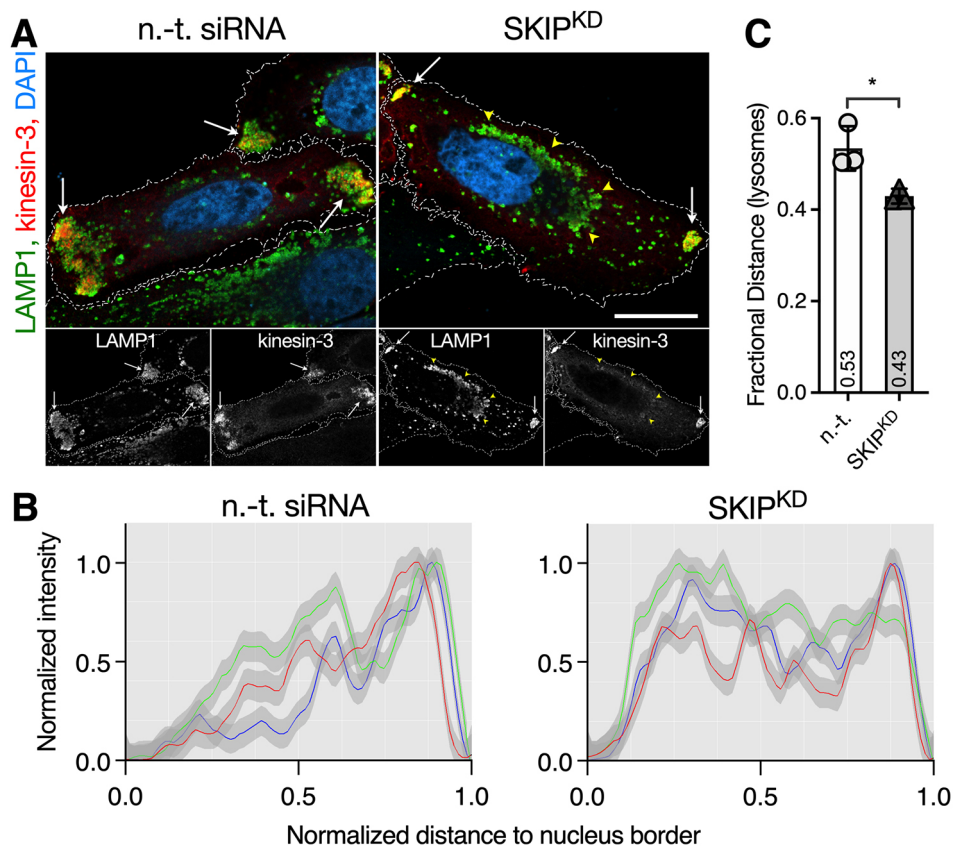
### SKIP is a player in the lysosomal recruitment of kinesin-3

Few studies have investigated the process by which kinesin-3 is recruited on lysosomes. This process is, however, known to involve interaction of the molecular motor with the Arl8a/b lysosomal GTPases (Wu et al., 2013). In the case of KIF1A, its activity has been shown to be independent of SKIP (Guardia et al., 2016). Based on this information, it was not easy to interpret the interaction we found between kinesin-3 and SKIP even though this interaction does not seem to be essential for the recruitment of kinesin-3 to SCVs. We thus decided to reconsider the role of Arl8a/b and SKIP in the activity of kinesin-3 on lysosomes. For this purpose, we analysed the distribution of lysosomes in HeLa cells overexpressing kinesin-3 and expressing or not expressing SKIP. Although the control cells were characterized by an almost total redistribution of the LAMP1 labelling at the cell periphery, in the absence of SKIP, we observed the frequent persistence of kinesin-3-negative lysosomes in the juxtannuclear area (Fig. 5A). A densitometric analysis of the distribution of the LAMP1 labelling between the edge of the nucleus and the plasma membrane clearly showed the

persistence of a lysosome population in the region close to the nucleus in cells not expressing SKIP (Fig. 5B). This resulted in a significantly lower FD for lysosomes in the absence of SKIP (Fig. 5C). These results therefore indicate the presence of lysosomal vesicles for which the recruitment of kinesin-3 requires SKIP, which had not been shown previously.

### SifA and Arl8a/b play a parallel role in the recruitment of kinesin-1 and kinesin-3

Arl8a/b GTPases are essential for lysosomal recruitment of molecular motors as they interact directly with kinesin-3 (Wu et al., 2013) and indirectly with kinesin-1 via SKIP (Rosa-Ferreira and Munro, 2011). Our current and previous results (Boucrot et al., 2005; Dumont et al., 2010) indicate that SifA shows the same interaction pattern with kinesins, suggesting that SifA and Arl8a/b have similar functions. To better understand the respective importance of these two parallel systems for the recruitment of kinesins, we used cells in which the expression of Arl8a/b was knocked down. We transfected HeLa cells with siRNA pools specific for one or the other GTPase (Fig. S7A), or with both siRNA pools (Fig. S7B) and observed in each case a very strong silencing of the expression of the targeted GTPases. Cells treated with a



**Fig. 5. The outward movement of lysosomes induced by kinesin-3 is partly dependent on SKIP.** HeLa cells were transfected with a non-targeting siRNA (n.-t.) pool or a siRNA against SKIP, and then with a plasmid for the expression of kinesin-3. They were then imaged by confocal microscopy. (A) Representative images for the localisation of lysosomes (LAMP1) and kinesin-3 in cells expressing or not SKIP. White arrows point to peripheral, kinesin-3-positive, lysosome clusters. Yellow arrowheads indicate the remaining kinesin-3-negative juxtannuclear lysosomes. Dashed lines show edges of cells. Scale bars: 20  $\mu$ m (main images; represents 40  $\mu$ m on the lower presentations). (B) Confocal images of kinesin-3-expressing cells, from three independent experiments, were quantified using the ImageJ Radial Profile plugin. The pooled profiles of 20 cells per experiment were used to plot the average intensity of LAMP1 labelling as a function of position in the cell. The graphs show the mean (smooth line)  $\pm$  the confidence interval (grey area) distribution of lysosomes for three independent experiments. In cells not expressing SKIP (SKIP<sup>KD</sup>) the lysosomes are distributed mainly in two peaks, one near the nucleus and the other near the plasma membrane. The first peak is not present in cells expressing SKIP (n.-t. siRNA) (C) The same images were used to calculate the mean FD for lysosomes. The data are means  $\pm$  s.d. of three independent experiments. \* $P < 0.05$  (unpaired two-tailed  $t$ -test used to compare the FDs).



cell periphery. However, in the presence of SifA and SKIP, we observed lysosome accumulations at the periphery identical to those observed in the control cells (Fig. 6A, bottom row, and 6D). In previous studies, we showed that the formation of these peripheral lysosomal clusters is linked to the recruitment of kinesin-1 by SKIP (Dumont et al., 2010). To test whether this is also true in the absence of Arl8a/b we expressed SifA and SKIP in cells in which kinesin-1 and GTPase expression were knocked down. We found that the lysosomes remained clustered in the juxtannuclear region (Fig. 6C,D). These results indicate that SifA is able to bypass the Arl8a/b function to mediate lysosomal recruitment of kinesin-1 via SKIP.

Overexpression of kinesin-3 in control cells triggered an outward movement of lysosomes and their accumulation together with the molecular motor at the periphery. These phenotypes were severely attenuated and the kinesin-3 located in the cytoplasm in the cells silenced for Arl8a/b, confirming the key role played by GTPases in the lysosomal recruitment of kinesin-3 (Fig. 6B,D). The co-expression of SifA made it possible to reverse these phenotypes, that is, to observe an accumulation of kinesin-3-positive lysosomes at the cell periphery, which the expression of SKIP did not achieve (Fig. 6B). The observations were identical in the presence of SifA<sup>L130D</sup>, indicating a SKIP-independent phenomenon (Fig. 6D). These results show that SifA recruits kinesin-3 independently of Arl8a/b.

Peripheral accumulation of lysosomes was observed in cells overexpressing SifA–SKIP and kinesin-3. This was accompanied in control cells by the presence of kinesin-3 on lysosomes, for which we observed little or no signal in the absence of Arl8a/b (Fig. 6B). As kinesin-1 is recruited by the SifA–SKIP complex (Dumont et al., 2007), competition between the two kinesins and preferential binding of kinesin-1 might explain this observation. To test this hypothesis, we used cells silenced for kinesin-1 and Arl8a/b. As mentioned above, the presence of SifA and SKIP in these cells did not modify the localization of lysosomes, which remained in the juxta-nuclear area. The additional overexpression of kinesin-3 resulted in peripheral clustering of lysosomes on which kinesin-3 was present (Fig. 6C,D).

All these results show that SifA and the SifA–SKIP complex recruit kinesin-3 and kinesin-1 and -3, respectively, in an Arl8a/b independent manner.

We finally infected Arl8a<sup>KD</sup>/b<sup>KD</sup> HeLa cells with *Salmonella* to test whether, in this context, SifA has the same autonomy in recruiting kinesins. In the absence of Arl8a/b, SITs were less frequent, an observation consistent with previous studies (Kaniuk et al., 2011; Moest et al., 2018). We also noted, under these conditions, a strong propensity of SCVs to migrate to the cell periphery suggesting a possible imbalance in the presence of molecular motors (Fig. S7C). Immunostaining revealed the presence of kinesin-1 on wild-type SCVs in the absence of GTPases, which was not the case in control cells (Fig. 7A,B). As a previous study had shown the presence of Arl8b is necessary for the recruitment of kinesin-1 to SCVs (Kaniuk et al., 2011), we also analysed cells infected with a *ΔsifA* mutant, which showed the unequivocal presence of kinesin-1 in both control and Arl8a/b-silenced cells (Fig. 7A,B). These results indicate that the recruitment of kinesin-1 on SCVs does not depend on either Arl8a/b or SifA. For kinesin-3, it seemed relevant to compare the recruitment on SCVs which display both Arl8a/b and SifA and on lysosomes on which only Arl8a/b are present. In cells infected with wild-type *Salmonella*, we observed, in the absence of Arl8a/b, a decrease of ~30% of the proportion of SCVs with kinesin-3 (56±2% versus

83±2% for the control; mean±s.d.), compared to a decrease of more than 80% on lysosomes (16±5% versus 85±7% for the control) in uninfected cells (Fig. 7C,D). These results show that, unlike lysosomes, recruitment of kinesin-3 to SCVs is largely independent of Arl8a/b.

## DISCUSSION

The T3SS-2 effectors play an important role in the infectious process of *Salmonella*. These bacterial proteins are transferred into the cytoplasm of infected cells, allowing them to mediate various biological processes in infected cells. The control of membrane exchange between the *Salmonella* vacuole and host cell compartments is of vital importance to the bacterium, and many effectors impact the shape, stability and membrane dynamics of *Salmonella* compartments. Among these, SifA recruits the host protein SKIP and the complex binds the molecular motor kinesin-1. The work presented in this study shows that the same complex also recruits kinesin-3 but in a different way.

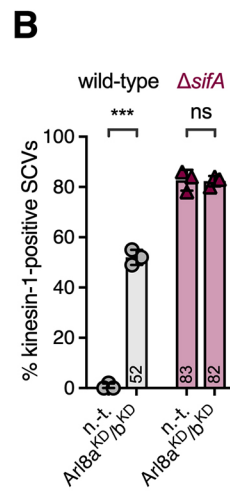
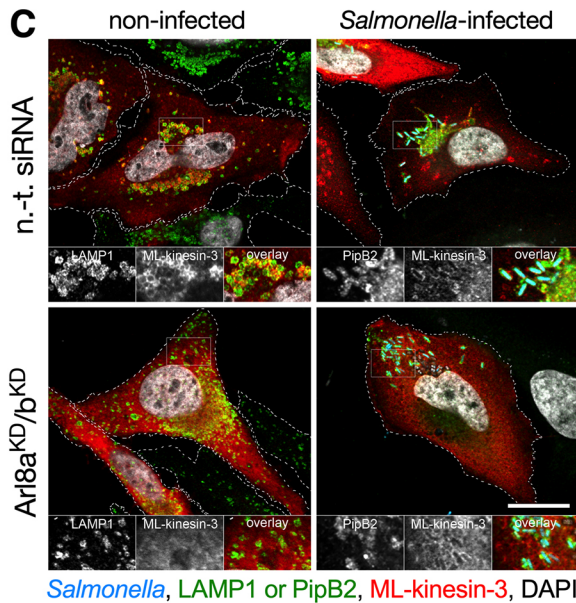
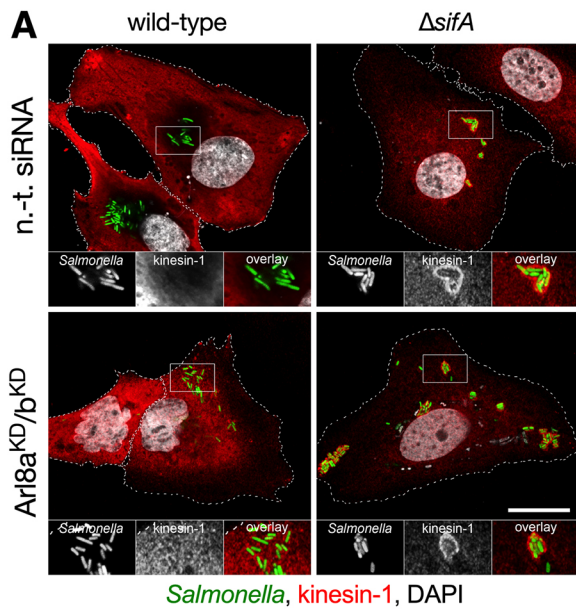
In this study, we used KIF1Bβ, which is a ubiquitous member of the kinesin-3 family (Matsushita et al., 2004). KIF1B is expressed as two main splicing variants (KIF1Bα and KIF1Bβ) that differ in their C-terminal portions and which transport different cargoes (Zhao et al., 2001). In contrast, the C-terminal domain of KIF1Bβ is very similar to that of KIF1A, another molecular motor in the kinesin-3 family, and both transport synaptic vesicle precursors (Okada and Hirokawa, 1999; Zhao et al., 2001) and lysosomes (Guardia et al., 2016). Here we show that, like KIF1Bβ, KIF1A is recruited to *Salmonella* compartments.

We produced antibodies against KIF1Bβ in rabbits which, by western blotting, demonstrated the presence and validated the knockdown of KIF1B in HeLa cells (Fig. S3A). However, these antibodies did not allow us to detect the endogenous protein by fluorescence microscopy. We therefore used overexpression of KIF1Bβ or a truncated form of the motor domain to define the conditions for kinesin-3 recruitment to SCVs and SITs. We screened different effectors involved in SIT formation for their contribution to kinesin-3 recruitment. Of these, only a *ΔsifA* mutant lacked the ability to recruit the molecular motor. The difference in recruitment between wild-type and *ΔsifA* SCVs is further enhanced after 4 h of infection. This period corresponds to the time window during which the T3SS-2 is established and SifA expressed (Beuzón et al., 2000). In contrast, *ΔssaV* SCVs differed from wild-type or *ΔsifA* SCVs as they had an intermediate level of kinesin-3 recruitment that only slightly evolved beyond 4 h of infection. In the absence of T3SS-2 effectors, these SCVs undergo phagolysosomal-like maturation through interaction with the late compartments of the endocytosis pathway (Beuzón et al., 2000) and consequently acquire kinesin-3.

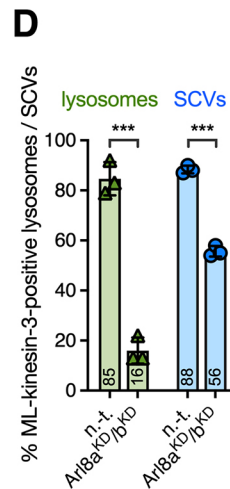
Co-immunoprecipitation experiments have shown that the SifA–SKIP complex is involved in the recruitment of kinesin-3 via interactions with SKIP and also with SifA. The experiments more precisely revealed interactions of the molecular motor with the N-terminal domain of SifA and the N- and C-terminal parts of SKIP. However, we have not been able to demonstrate these interactions with endogenous kinesin-3 in either co-immunoprecipitation assays or pulldown experiments using purified recombinant forms of SifA or SKIP (Boucrot et al., 2005; Diacovich et al., 2009). The origin of this negative result is difficult to determine but is most likely related to the low level of kinesin-3 expression in HeLa cells.

Despite this, kinesin-3 or kinesin-1 silencing has clear consequences on three SifA- and/or SKIP-dependent criteria in infected cells, namely the positioning and membrane dynamics of





**Fig. 7. Recruitment of kinesin-1 and kinesin-3 to *Salmonella* compartments is essentially independent of Arl8a/b.** HeLa cells were transfected with a non-targeting siRNA (n.-t.) pool or siRNA pools for the knockdown of Arl8a/b. These cells were further transfected with plasmids for kinesin-1 or ML-kinesin-3 expression, and infected or not for 16 h. (A) Confocal images show that the recruitment of kinesin-1 to  $\Delta sifA$  SCVs is independent of Arl8a/b. Note that kinesin-1 labelling is also visible on wild-type SCVs in Arl8a<sup>KD</sup>/b<sup>KD</sup> cells, unlike control cells. (B) The percentages of kinesin-1-positive SCVs were determined by epi-fluorescence microscopy. (C) Confocal images of cells uninfected or infected with wild-type *Salmonella* and immunostained for LAMP1 or PipB2 and kinesin-3. In contrast to lysosomes, the recruitment of ML-kinesin-3 to SCVs is mostly independent of Arl8a/b. (D) The percentages of ML-kinesin-3-positive lysosomes and SCVs were determined by epi-fluorescence microscopy. In A and C, dashed lines show edges of cells. Scale bars: 20  $\mu$ m (main images; represents 10  $\mu$ m on the magnified views). Data in B and D are means  $\pm$  s.d. of three independent experiments. Not significant (ns),  $P > 0.05$ ; \*\*\* $P < 0.001$  (two-way ANOVA and Šídák multiple comparisons tests were used to compare the results in knocked-down and control cells).



*Salmonella* compartments, and the intracellular replication of the bacteria. We observed a very strong decrease in the number and important alterations in the length and thickness of SITs in the absence of kinesin-3. With regard to kinesin-1, its absence resulted in thick SITs that were frequently wrapped around the nucleus (Fig. 2). Finally, the knockdown of both kinesins only accentuated the phenotype observed in the absence of kinesin-3, that is, a very marked decrease in the number of SITs, with those remaining mostly short and thin. These results suggest that the action of kinesin-3 is essential for the biogenesis of SCV-associated tubules and precedes that of kinesin-1. Kinesin-1 seems to be more involved in centrifugal elongation of tubules, as previously shown (Knodler and Steele-Mortimer, 2005). Kinesin-1 and -3 preferentially bind to microtubules with different post-translational modifications. Kinesin-1 preferentially binds acetylated microtubules, which are located in the perinuclear region. Kinesin-3 preferentially binds tyrosinated microtubules, which are located more radially and

peripherally (Guardia et al., 2016). These data are clearly difficult to reconcile with our knockdown results, which indicate kinesin-3 activity in the juxta-nuclear region and kinesin-1 activity on radial microtubules. However, it is likely that the affinities of one or other of the kinesins for these two types of microtubules and the localizations of the microtubules are all relative and that other factors in the host, and also in the bacterium, modulate this theoretical pattern.

Increased instability of SCVs was observed in the absence of kinesin-3. This phenomenon is observed in the late stage of infection in cells infected with a  $\Delta sifA$  mutant (Beuzón et al., 2000). Since the absence of other SifA-interacting proteins, such as SKIP (Boucrot et al., 2005), PLEKHM1 (McEwan et al., 2015) or Arl8b (Kaniuk et al., 2011; Moest et al., 2018), has not been reported to impact the stability of SCVs, the lack of recruitment of kinesin-3 by the  $\Delta sifA$  mutant may well underlie the phenotype. One of the consequences of this instability and the disruption of SCVs is the

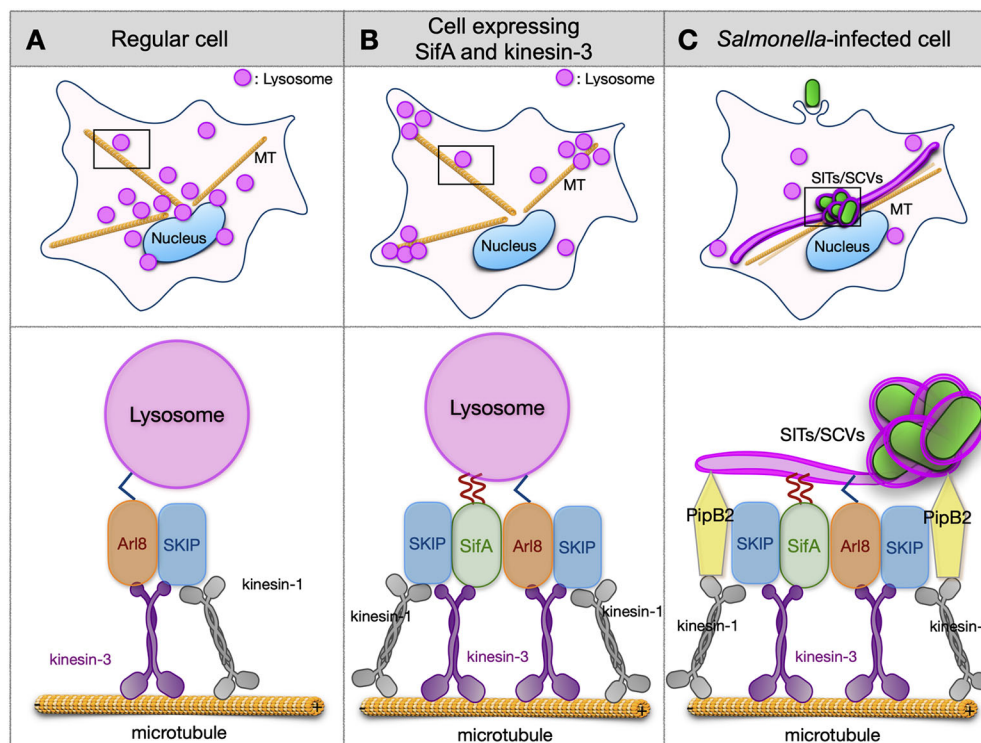
presence of bacteria in the cytosol of epithelial cells and a hyper-replication of *Salmonella* (Knodler et al., 2014). However, the absence of kinesin-3, unlike SKIP, has no impact on the intravacuolar replication. Yet it is very likely that in phagocytic cells that do not support cytoplasmic replication of *Salmonella*, or *in vivo*, that the absence of kinesin-3 would have an overall negative impact on intracellular replication and virulence of *Salmonella*, as has been shown for the absence of SifA (Beuzón et al., 2000). SITs facilitate membrane exchange between the bacterial and host compartments (Schroeder et al., 2010), and thereby promote nutrient supply and intravacuolar bacterial multiplication (Liss et al., 2017). The significant degradation in the number and structure of SITs observed in the absence of any of the kinesins tested did not, however, alter the intravacuolar replication of *Salmonella*. The residual SITs are therefore sufficiently dense and functional to ensure nutrition of the bacteria. They are, however, ineffective, at least in the absence of kinesin-3, in maintaining the integrity of SCVs. The two phenotypes thus seem to be dissociated and the results are all the more puzzling, as the SCVs were found to be stable in the absence of kinesin-1 and -3, whereas the formation of SITs is strongly restricted under these conditions.

Finally, our results also show the impact of these kinesins on the positioning of SCVs. The inhibition of kinesin-3 expression position SCVs closer to the nucleus. The activity of this molecular motor thus keeps the SCVs in the peri-nuclear region but at a distance from the nucleus, an observation we had previously made about kinesin-1 in infected macrophages (Alberdi et al., 2020).

In uninfected cells, the complex of Arl8a/b and SKIP is an essential player in the lysosomal recruitment of kinesin-3 and kinesin-1. Kinesin-3 interacts with Arl8a/b (Wu et al., 2013) and SKIP (our results), and kinesin-1 with SKIP (Boucrot et al., 2005). From this point of view, our study establishes an organizational parallel between Arl8a/b and SifA given that it shows that these proteins have the same interaction pattern with kinesin-1 and -3 (Fig. 8). It also establishes a functional parallel by showing that ectopic expression of

SifA in HeLa cells bypasses the role of Arl8a/b for the recruitment and activity of both kinesins. Since Arl8b is, like SifA, present on SCVs and SITs (Kaniuk et al., 2011), there are two parallel and interacting (Moest et al., 2018) kinesin recruitment systems in infected cells. That said, it is necessary to balance these assertions on a few points. The first concerns the localization of the lysosomes which, when SifA or Arl8a/b are overexpressed alone, tend to concentrate in the juxta-nuclear region with the former or in the periphery with the latter (Hofmann and Munro, 2006). The explanation for this difference may lie in the recent discovery of the role of Arl8 proteins in the activation of SKIP, allowing the recruitment of kinesin-1, which SifA may not be able to do or could do differently (Keren-Kaplan and Bonifacio, 2021). The second point concerns the recruitment of kinesin-1 to *Salmonella* compartments. This is also carried out by PipB2, another effector of T3SS-2 (Henry et al., 2006) (Fig. 8). Thus, kinesin-1 is indeed present on SCVs independently of the presence of SifA and/or Arl8a/b, contrary to what has been published (Kaniuk et al., 2011). Finally, our results revealed an interaction between kinesin-3 and SKIP. These results were quite unexpected as previous work had shown that KIF1A activity is independent of SKIP (Guardia et al., 2016). Our data indicate the existence of a population of lysosomes insensitive to the action of kinesin-3 in the absence of SKIP, thus confirming the importance of this interaction in the positioning of lysosomes. However, in infected cells, SifA binds kinesin-3 almost as well as the SifA-SKIP complex (Fig. 3E), indicating that SKIP is not essential for the recruitment of kinesin-3 to the *Salmonella* compartments.

This study shows that SifA secretion in *Salmonella*-infected cells establishes a kinesin-3 recruitment system on the surface of the *Salmonella* compartments that is parallel to that of Arl8a/b and is at least as efficient as the latter. Much remains to be discovered about the respective roles of kinesin-1 and kinesin-3 and their relationship with the proteins that enable their membrane recruitment. In infected cells, it will be necessary to understand whether, as our data suggest, and how kinesin-3 initiates the formation of SITs. In the



**Fig. 8. Model of kinesin-1 and kinesin-3 recruitment in different cellular contexts.** (A) N-terminal acetylation of Arl8 (Hofmann and Munro, 2006) allows its association with lysosome membranes. Arl8 recruits kinesin-1 indirectly via SKIP (Boucrot et al., 2005) and kinesin-3 by direct interaction (Wu et al., 2013) and via SKIP (this study). (B) SifA is C-terminally lipidated (Boucrot et al., 2003; Reinicke et al., 2005), which allows its anchoring to lysosomes upon ectopic expression. Like Arl8, SifA recruits kinesin-3 (this study) and kinesin-1 via SKIP (Boucrot et al., 2005). Co-expression of SifA and kinesin-3, or SifA and SKIP causes outward movement of lysosomes, even in the absence of Arl8 proteins. (C) In *Salmonella*-infected cells, SifA and Arl8 proteins interact (Moest et al., 2018) and establish two parallel recruitment systems for kinesin-1 and -3 on SCVs and SITs. However, kinesin-1 recruitment is primarily mediated by the T3SS-2 effector PipB2 (Henry et al., 2006 and this study) and kinesin-3 recruitment is largely independent of Arl8 (this study).



non-infected condition, it would be important to know whether the lysosomes that are insensitive to kinesin-3 activity in the absence of SKIP constitute a distinct compartment of endocytosis and how SKIP, which is itself recruited by Arl8a/b, can be a discriminant for kinesin-3 recruitment.

## MATERIALS AND METHODS

### Antibodies and reagents

The antibodies used in this study were: mouse anti-FLAG (clone M2, Sigma, 1:500), mouse anti-GFP (JL-8, Takara, 1:500), mouse anti-actin (AC40, Sigma, 1:1000), mouse anti-LAMP1 (H4A3, 1:1000), rabbit anti-LAMP1 (antibody was made in-house and was raised against the cytoplasmic domain of LAMP1, 1:1000), mouse anti-Myc (clone 9E10, made in-house, 1:1000), mouse anti-HA (clone 16B12, Covance, 1:1000), mouse anti- $\beta$ -tubulin (clone TUB 2.1, Sigma, 1:1000), rat anti-HA (clone 3F10; Roche Molecular Biochemicals, 1:500), rabbit polyclonal anti-Arl8a (Proteintech, #17060-1-AP, 1:200), rabbit polyclonal anti-Arl8b (Proteintech, #13049-1-AP, 1:200), goat anti-mouse- or anti-rabbit IgG coupled to peroxidase (Sigma, 1:10,000), and donkey anti-rat- or anti-mouse IgG conjugated to Alexa Fluor (488, 546 or 647) (Jackson ImmunoResearch, 1:500). The mouse anti-(human) LAMP1 H4A3 monoclonal antibodies developed by J. T. August were obtained from Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa (Department of Biology). The rabbit anti-KHC (KIF5B, PCP42) was generously provided by Ronald D. Vale (University of California, San Francisco, CA).

The (His)<sub>6</sub> and GST-tagged polypeptides corresponding to KIF1B $\beta$  (amino acids 1324–end) were expressed as BL21(DE3), purified from bacterial lysates on HisTrap<sup>TM</sup> HP (GE Healthcare) or glutathione–Sepharose 4B beads (Pharmacia Biotech), respectively, following the manufacturer's instructions and stored at –80°C in PBS with 20% glycerol. Purified (His)<sub>6</sub>-KIF1B $\beta$  (1324–end) was used to immunise rabbits (four intradermal injections of 50  $\mu$ g recombinant protein per rabbit, spaced 21 days apart). Rabbits were collected after 90 days. Anti-kinesin-3 antibodies were purified from serum on purified GST–KIF1B $\beta$  (1324–end) immobilised on PVDF after SDS-PAGE and electrotransfer (Fang, 2012).

Unless otherwise stated all other reagents were purchased from Sigma-Aldrich (St Louis, MO).

### Cell lines and culture conditions

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

### Bacterial strains, eukaryotic cells infection and immunofluorescence

*Salmonella enterica* serovar Typhimurium and isogenic mutant strains used in this study are listed in Table S3. Strains were cultured in LB broth (Difco) at 37°C. Ampicillin (50 mg/ml), kanamycin (50 mg/ml) and chloramphenicol (50 mg/ml) were added when required. HeLa cell infections and immunofluorescence were carried out as described previously (Schroeder et al., 2010). DNA was stained by mounting the coverslips on glass slides with ProLong<sup>®</sup> Gold antifade reagent with DAPI (Invitrogen).

### Molecular biology

The plasmids and oligonucleotides used in this study are described in Tables S1 and S2, respectively. The plasmids were constructed by Gateway recombination cloning technology. All the information concerning the PCR products (oligo-nucleotides, template), the donor vector, the destination vector and the final plasmids are presented in Tables S1 and S2.

### Transfection of cells

HeLa and COS-7 cells were transfected using FuGENE<sup>®</sup> 6 following the manufacturer's protocol. For knockdown experiments, cells were

transfected with 25 or 50 nM single siRNA (against SKIP; Boucrot et al., 2005) or siRNA pools (against KIF5B, KIF1B $\beta$ , Arl8a and/or Arl8b) using jetPRIME<sup>®</sup> (Polyplus transfection) following the manufacturer's protocol, and analysed 72 h later. Non-targeting siRNA (D-001810-10-05) and ON-TARGETplus SMARTpool siRNAs targeting KIF5B (L-008867-00), KIF1B (L-009317-00), Arl8a (L-016577-01) and Arl8b (L-020294-01) were purchased from Dharmacon.

### Co-immunoprecipitation

Transfected HeLa cells were washed with PBS, scraped with a rubber policeman, centrifuged for 5 min at 400 g and resuspended in 200  $\mu$ l lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA and 0.5% NP-40) supplemented with a protease inhibitor cocktail. After 30 min at 4°C, cell lysates were diluted with 300  $\mu$ l dilution buffer (lysis buffer without NP-40) and centrifuged at 20,000 g for 10 min at 4°C. 10% of the lysates were boiled in Laemmli sample buffer. Immunoprecipitations were performed with GFP-Trap<sup>®</sup> beads (ChromoTek GmbH) that contain a recombinant alpaca anti-GFP antibody covalently coupled to agarose beads following manufacturer's protocol. Immunoprecipitated proteins were analysed by SDS-PAGE and western blotting using appropriate antibodies as described previously (Méresse et al., 1995).

### Fractional distance analysis

We have written a macro in Fiji (a distribution of the free software ImageJ; Schindelin et al., 2012) to define and quantify the position of the organelles relative to the nucleus and the plasma membrane. After opening an image file, the macro first asks the user to draw the contours of the nucleus and the cell and to save their radial profile (Paul Baggethun's Radial Profile plugin). The normalised part of this plugin has been removed in order to consider the real distribution of organelles. From these profiles, the following parameters are calculated: the maximum radius of the cell (R<sub>c</sub>) and the maximum radius of the nucleus (R<sub>n</sub>). In a second step, the user specifies a minimum intensity threshold to select the organelles (in this study bacteria) and the radial profile is also recorded. Using the Euclidean distance map (EDM) of the nucleus contour, the organelles mean distance to nucleus border (D) is extracted. The fractional distance, is the normalization of this value between 0 and 1 obtained by dividing D by R<sub>c</sub> minus R<sub>n</sub>.

To plot the distribution of lysosomes as a function of distance from the edge of the nucleus, the values of X and Y were normalised between 0 and 1 and 20 profiles from the same experiment were pooled into a single profile. This grouped profile was used to build a smooth line to identify the trend for each experiment. For this purpose, an R script (<https://www.R-project.org/>) was used. The smooth line and the confidence interval were generated using the ggplot2 library with the Loess ('local weighted smoothing') method, a commonly used tool in regression analysis.

### Statistical analyses

Statistical analyses were performed with Prism 9 software (GraphPad).

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: Z.F., T.M., S.M.; Methodology: Z.F., M.F., S.M.; Software: M.F.; Validation: Z.F., S.M.; Formal analysis: M.F.; Investigation: T.M.; Writing - original draft: S.M.; Writing - review & editing: J.-P.G., S.M.; Supervision: J.-P.G., S.M.; Funding acquisition: J.-P.G., S.M.

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