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Live imaging reveals *Listeria* hijacking of E-cadherin recycling as it crosses the intestinal barrier

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SUMMARY

Listeria monocytogenes is a foodborne bacterial pathogen that causes human listeriosis, a severe systemic infection [1]. Its translocation across the intestinal epithelium is mediated by the interaction of internalin (InlA), a *Listeria* surface protein, with its host species-specific receptor E-cadherin (Ecad) [2-5]. It occurs through goblet cells, on which Ecad is lumenally accessible [6], via an unknown mechanism. In the absence of cell lines recapitulating this phenotype *in vitro*, we developed an *ex vivo* experimental system, based on the intraluminal microinjection of *Listeria* in untreated, pharmacologically-treated and genetically modified intestinal organoids. Using both live light sheet microscopy and confocal imaging, we show that *Listeria* translocates through goblet cells within a membrane vacuole in an InlA- and microtubule-dependent manner. As Ecad undergoes constant apical-basal recycling [7, 8], we hypothesized that *Lm* may transit through goblet cells by hijacking Ecad recycling pathway. Indeed, *Listeria* is stuck at goblet cell apex when Ecad endocytosis is blocked, and remains trapped intracellularly at the basolateral pole of goblet cells when Rab11-dependent Ecad recycling is compromised. Together, these results show that *Listeria*, upon docking onto its lumenally accessible receptor Ecad, hijacks its recycling pathway to be transferred by transcytosis across goblet cells. Live imaging of host-pathogen interactions in organoids is a promising approach to dissect their underlying cell and molecular biology.

Keywords

Listeria, epithelial translocation, organoids, intestine, goblet cell, E-cadherin recycling, live imaging, Rab11

RESULTS AND DISCUSSION

In order to decipher the cell biology mechanisms of *Listeria* translocation across the intestinal epithelium, we developed a genetically amenable experimental system permissive to InlA-Ecad-dependent trans-epithelial crossing. Deciphering the detailed cell biology mechanisms of *Listeria* translocation across the intestinal epithelium *in vivo* would require interfering with cellular pathways that may disrupt its barrier function. Additionally, it would be extremely challenging to capture *Listeria* translocation in real-time across intestinal villus epithelium, given its rarity and intestinal peristalsis. Furthermore, no adherent cell, including human colonic cell lines T84, HT29 and Caco-2, displays an apical-basal polarization and apical accessibility of Ecad, which are both critical for *Listeria* InlA-Ecad-dependent trans-epithelial crossing to occur (our unpublished observations). We therefore set up an *ex vivo* experimental system based on the microinjection of *Listeria* in the lumen of intestinal organoids. Intestinal organoids derive clonally from intestinal stem cells, which give rise to a fully differentiated, polarized intestinal epithelium that forms a so-called “minigut” centered by a lumen, and contains differentiated intestinal cell subtypes [9]. Intestinal organoids are genetically amenable [10-13], can be subjected to pharmacological interventions [14], and can also be imaged both fixed and alive [9, 15].

Intestinal organoids contain goblet cells

We generated intestinal organoids from the small intestine of knock-in E16P (E16P KI) mice, in which the endogenous mouse Ecad is punctually modified to express a proline at position 16 of the mature protein in place of a glutamic acid. This modification enables this “humanized” mouse Ecad to interact with InlA and mediate *Listeria* internalization [1, 4, 16] (see Methods). As expected [9], E16P organoids grown in Matrigel[®] exhibit a fully mature apical-basal polarity and display cell subtype heterogeneity. Organoids intestinal stem cells differentiate into enterocytes (Villin⁺), enteroendocrine cells (ChromograninA⁺), goblet cells (WGA⁺/Lysozyme^{low}) and Paneth cells (WGA^{low}/Lysozyme⁺, Figure S1A). Additionally, and as previously reported [17], intestinal organoids do not contain M cells (GP2⁺) (Figure S1A). As *Listeria* cross the intestinal barrier via goblet cells *in vivo*, it is important to unambiguously identify this cell subtype in our experimental model. Goblet cells are mucus-secreting cells with a characteristic goblet-shaped cell morphology, which nucleus location does not align with neighboring enterocytes [18]. Additionally, goblet cells can be identified by immunolabeling of the Muc2 mucin, a major mucus component, following Carnoy hydrophobic fixation [19]. However, Muc2 labeling cannot be applied to our experimental

system: Carnoy fixation damages plastic wells in which Matrigel® embedded organoids are located, and more importantly, renders Matrigel® opaque. Therefore, we used wheat germ agglutinin (WGA), which labels mucus by binding to sialic acid and N-acetyl-glucosaminyl carbohydrate residues on mature, modified mucins [20, 21]. WGA positive cells were co-labeled for cytokeratin-18, which is specifically expressed in goblet cells in the gut [22] (Figure S1B). Of note, PFA fixation can dissolve mucus, resulting in bona-fide WGA-negative goblet cells. Therefore, we used the following criteria to identify goblet cells: (i) WGA labeling, (ii) goblet-shaped cellular morphology, and (iii) misaligned nucleus relative to neighboring cells. When WGA-negative cells strictly met the last two conditions, we also considered them as goblet cells (e.g., in Figure S1D right).

***Listeria* translocation across intestinal organoid goblet cells requires InlA**

We microinjected 5×10^3 CFUs of wild type *Lm* into the lumen of mature organoids and investigated bacteria interactions with the intestinal epithelium (Figure 1A). Confocal imaging of intestinal organoids fixed 1 hour after microinjection allowed the detection of bacteria both inside and underneath the basolateral pole of goblet cells (Figure 1B, Video S1). Quantification studies 16 hours post-infection revealed that bacteria were all located exterior to microinjected organoids, specifically associated with goblet cells, as a result of translocation events (Figure 1C). Of note, the microinjection procedure and/or presence of bacteria in the organoid lumen did not modify the proportion of WGA⁺ cells (10.87 ± 3.07 %) compared to non-injected organoids (9.56 ± 2.62 %, Figure S1C). Consistent with our previous *in vivo* studies in humanized mice permissive to InlA-Ecad interaction [5, 6, 16], this phenotype was strictly InlA-dependent. In contrast, it was independent of LLO and ActA, which mediate *Lm* escape from its internalization vacuole and actin-based motility, respectively (Figure 1C). *Li*(InlA) is derived from *L. innocua*, a non-pathogenic *Listeria* species devoid of *L. monocytogenes* virulence factors, which has been genetically modified to express InlA, and enters into cells in an Ecad-dependent manner [23]. Upon microinjection in intestinal organoids lumen, *Li*(InlA) was also located extracellularly at basolateral pole of goblet cells, as wild type *Lm* (Figure 1C, Figure S1D,E, Video S1). This indicates that *Listeria* crossing of the intestinal epithelium does not require escape from the vacuole and actin-based motility. In some cases, bacteria underneath goblet cells were surrounded with WGA-labeled material, implying that bacteria can translocate with mucus (Figure S1E). Of note, bacteria beneath goblet cells cannot replicate or move within Matrigel®, as it contains gentamicin and is bactericidal. Together, these results show that *Listeria* microinjection in the

lumen of intestinal organoids allows to faithfully recapitulate *Listeria* translocation across the intestinal epithelium *in vivo*. These results also confirm that InlA is necessary and sufficient to mediate *Listeria* translocation across goblet cells, while LLO and ActA are dispensable [6], and justify the use of *Li*(InlA) for further experiments, in order to minimize the potential cytotoxicity of LLO to microinjected organoids [24].

Real-time imaging of *Listeria* transcytosis across intestinal organoid epithelium

Despite converging evidences indicating that *Listeria* transcytoses through goblet cells *in vivo* [6], live imaging has not been performed to prove it actually occurs, *i.e.* translocation of a bacterium surrounded by its internalization vacuole. We therefore set up experimental conditions to image in real time translocation of *Li*-GFP(InlA) across intestinal organoids in which cell membranes are constitutively red fluorescent (mtd-Tmt; E16P KI). *Listeria* translocation across the intestinal epithelium is a rare event *in vivo*, as only 3 to 4% of intestinal villi are infected in 45 minutes long intestinal ligated loop assays (our unpublished observation). Moreover, *Lm* translocation across the small intestinal epithelium occurs within 30 minutes *in vivo* [6]. The rarity of translocation events requires that entire organoids are scanned to be captured, and this may exceed the time needed for bacterial translocation when using classical confocal imaging. Additionally, laser power has to be minimal to preserve the fluorescence signal of individual bacteria and cell membrane over prolonged imaging. Furthermore, imaging has to be performed at the adequate spatial resolution to track micron-sized bacteria within intestinal organoids, the diameter of which ranges from hundreds to thousands of microns. To accommodate all these experimental requirements, light sheet microscopy was used, which can image intestinal organoids at least thirty times faster than a regular confocal microscope with minimal phototoxicity. 5×10^3 CFUs of *Li*-GFP(InlA) were microinjected intraluminally into mtd-Tmt; E16P KI organoids. Real time imaging revealed that a bacterium reached cell basolateral pole surrounded by a cell membrane in less than 12 minutes. During the following 36 minutes, the bacterium remained at the basolateral pole of the cell, surrounded by mtd-Tmt signal, and then exited from the cell basolaterally (Figure 1D, Video S2). This allowed to image for the first time *Listeria* transcytosis across the intestinal epithelium directly and unambiguously.

Ecad is lumenally accessible on goblet cells in intestinal organoids

InlA-dependent translocation of *Listeria* specifically through goblet cell (Figure 1B,C, Figure S1D,E) suggests that translocation is mediated by InlA interaction with lumenally accessible

Ecad on goblet cells, as it has been observed *in vivo* [6]. To assess luminal accessibility of Ecad in organoids, we applied an Ecad antibody directed against its ectodomain (ECCD-2) to the accessible luminal side of fixed and sectioned organoids (Figure 1E). To stain only accessible Ecad, we performed surface immunolabeling without cell permeabilization. Junctional proteins in the sectioned plane are exposed, accessible without permeabilization, and are therefore also labeled. However, junctional proteins located below the cutting plane cannot be labeled in the absence of cell permeabilization, unless they are inherently lumenally accessible (Figure 1E,F). As expected given the specific location of InlA-Ecad *Listeria* translocation (Figure 1C), lumenally accessible Ecad was detected only on the apical side of mucus-expelling goblet cells in non-permeabilized organoids (Figure 1E, Video S3). In contrast, in permeabilized organoids, Ecad was detected at *adherens* junctions, down to the basolateral membrane in all cells regardless of the subtype (Figure 1F, Video S3). Together, these results indicate that the cells on which Ecad is lumenally accessible in intestinal organoids are mucus-expelling goblet cells, and that bacteria located beneath the basal pole of goblet cells after intraluminal microinjection have undergone InlA-Ecad-mediated transcytosis. This establishes the specificity of this *ex vivo* experimental system to study the cell biology of *Listeria* InlA-Ecad translocation across the intestinal epithelium.

Endocytosis and microtubule dynamics are required for *Listeria* translocation through goblet cells

Previous *in vivo* investigations have shown that InlA-Ecad-dependent *Listeria* crossing of the intestinal barrier depends on microtubule and the exocytic machinery [6]. We therefore hypothesized that *Listeria*, upon its docking on lumenally accessible Ecad on goblet cells, hijacks Ecad recycling pathway to cross the intestinal epithelial barrier, from its dynamin-mediated endocytosis [7] and endosomal trafficking along microtubules [25], to its Rab11 dependent release at the cell basolateral pole [26, 27]. Ecad recycling has not been studied in goblet cells, but it is also expected to involve its endocytosis at the apical pole and basolateral recycling in a microtubule-dependent manner. We therefore tested this hypothesis by dissecting the role of Ecad recycling pathway on *Listeria* transcytosis.

In polarized differentiated cells, Ecad, which forms *adherens* junctions (AJ), is endocytosed in a clathrin-dependent manner [7]. The resulting endosomes are excised from the plasma membrane by the GTPase dynamin [28, 29]. Ecad is trafficked in a microtubule-dependent manner [25, 30, 31] and recycled to the basolateral membrane [7]. To first inhibit Ecad endocytosis, we used dynasore, a dynamin inhibitor that prevents the fission of clathrin- and

caveolin-dependent endocytic vacuoles [32, 33]. To inhibit microtubule-based Ecad trafficking, we used colchicine, which blocks microtubule polymerization. In organoids treated 2 hours with dynasore, cytosolic endocytic Ecad *punctae* were drastically reduced (Figure S2C), as previously reported in cultured cells [29]. In presence of colchicine, more cells with metaphase-blocked mitoses were observed (Figure 2A) as expected [34], indicating that both drugs are active in our system. Nevertheless, the overall morphology and polarity of the organoids were comparable to non-treated controls (Figure 2A) and we observed no difference in total bacterial association to cells (either apically, intracellularly or basolaterally) (Figure 2B). Yet there was a significant decrease of extracellular translocated bacteria in dynasore- and colchicine-treated organoids, relative to control organoids (Figure 2C). Consistent with a similar total bacterial association to cells in all conditions, accessible Ecad was detected similarly on goblet cells in dynasore- and colchicine-treated conditions (Figure 2D, Figure S2, Video S4), as in untreated organoids (Figure 1E). This suggests that the interaction between *Listeria* and its receptor Ecad is not impaired in dynasore- and colchicine-treated organoids, whereas *Listeria* transcellular transport is blocked within goblet cells. To examine where bacteria were trapped in cells, we investigated their location in three distinct compartments: apically associated to the cell membrane, intracellular, and extracellular at their basal pole. In dynasore-treated organoids, bacteria were mostly apically associated (Figure 2C,E dynasore, Figure S3, Video S4). These bacteria were likely trapped in elongated invaginations of the plasma membrane, as observed both in *Drosophila* and mammalian epithelial cells when dynamin is inhibited [35-37]. In colchicine-treated organoids, where microtubule dynamics is inhibited, intracellular bacteria were located in the median part of the cells (Figure 2C,E colchicine, Figure S3, Video S4). In all experimental conditions, intracellular bacteria were surrounded with Tomato red-labelled membrane, indicating that bacteria were within a vacuole (Figure 2E). Together, these results show that upon InlA-mediated *Listeria* docking to Ecad, Ecad endocytosis is required to complete bacterial internalization into goblet cells, and microtubules dynamics is required for bacterial trans-epithelial transit in a vacuole.

Rab11 is required to complete *Listeria* intestinal translocation through goblet cells

We next investigated whether recycling of Ecad endosomes is required for *Listeria* InlA-dependent translocation across the intestinal epithelium. Ecad is recycled and trafficked *via* Rab11-positive recycling endosomes, which recruit the exocytosis machinery [8, 26, 27, 38, 39]. Rab11 is also involved in basolateral sorting of newly synthesized Ecad in polarized

mammalian cells *in vitro* [38]. The kinetics of Rab11-based trafficking is around 30 minutes [40, 41], which fits with our live imaging of *Listeria* crossing the epithelium (Figure 1D and Videos S2). Therefore, to perturb Ecad recycling, we targeted Rab11. Since Rab11 null mutations are embryonically lethal in mice [42, 43] and constitutive knock-out of Rab11 may be detrimental for the development of intestinal organoids, we generated lentivirus-transduced organoids in which a dominant negative and myc-tagged version of Rab11 can be induced by doxycycline. As Rab7-dependent Ecad degradation pathway [44] is not expected to be involved in bacterial translocation, we also generated lentivirus-transduced organoids in which a dominant negative Rab7 can be induced, to use them as negative controls. To visualize successful transduction and transcription induction, we used a bi-cistronic reporter system where an internal ribosome entry site (IRES) is placed downstream of each DN mutant and upstream of a mCerulean coding sequence (Figure 3A,B). Organoids were grown and maintained in presence of antibiotics to select for transduced cells. When organoids were properly formed and mature, doxycycline was added 12-16 hours before microinjection to induce the transcription of dominant negative Rab11 and Rab7 variants Rab11DN and Rab7DN, respectively (Figure 4A). Organoids expressing Rab11DN exhibited cytosolic Ecad *punctae* that accumulated at basolateral side of epithelial cells compared to control organoids, consistent with a blockade of Ecad release when Rab11 is non-functional (Figure S2C). Induced expression of Rab7DN caused general enrichment of cytosolic Ecad (Figure S2C), and large Ecad aggregates formed throughout the apical-basal axis (Figure S2C), suggesting that Ecad degradation is affected when Rab7 is non-functional [44]. However, inducing Rab11 and Rab7 dominant negative proteins for a short period of time (less than 24 hours) did not detectably impair epithelium morphology (Figure 3C) and Ecad luminal accessibility compared to WT organoids (Figure 4B, Figure S2, Videos S5). Microinjection experiments revealed that induction of Rab7DN did not affect bacterial translocation through goblet cells. *Listeria* was found extracellular at the basolateral pole of goblet cells of Rab7DN-induced organoids, to the same degree than in transduced but non-induced control organoids (NI). This shows that bacteria complete translocation normally within goblet cells in presence of Rab7DN (Figure 4C-E Rab7DN, Figure S3, Video S5). In contrast, in mycRab11DN-induced transduced organoids, a significant decrease of translocated bacteria beneath goblet cells was observed, together with a corresponding significant increase of intracellular bacteria. Intracellular bacteria were mostly located at the basolateral pole of goblet cells (Figure 4C-E Rab11DN, Figure S3, Video S5). This was also the case for wild type *Listeria* microinjected in mycRab11DN-induced organoids, even 16-

hour post microinjection (Figure S2D-F). Note that prolonged exposure to doxycycline might have prevented *Listeria* vacuolar escape and cell-to-cell spread at this late time point. Taken together, these data show that Rab11, in contrast to Rab7, is required for the release of bacteria from the basal pole of goblet cells, highlighting that *Listeria* hijacks Rab11-dependent Ecad recycling for trans-epithelial translocation *via* goblet cells.

Organoid as a model for real-time investigation of host-pathogen interactions

Here, we have developed an *ex vivo* minigut system in which *Listeria* crosses the intestinal barrier by transcytosis through goblet cells as it does *in vivo* [6]. We have harnessed the power of this experimental system to directly image, in real time, the trans-epithelial translocation of a microbial pathogen, and to dissect the underlying cell biology and molecular mechanisms. *Listeria* crossing of the intestinal barrier relies on the specific interaction of InlA with lumenally accessible Ecad at the apical pole of goblet cells. We have shown that (i) bacterial Ecad-dependent internalization in goblet cells requires dynamin-mediated endocytosis, (ii) bacteria transit through these cells in a microtubule-dependent manner as *in vivo* [6], and (iii) Rab11 is required for the release of *Listeria* at the basolateral pole of goblet cells, thereby showing that *Listeria* hijacks E-cadherin recycling pathway to cross the intestinal barrier (Figure S3). While organoids have been used to investigate infection with human norovirus and SARS-CoV-2 [45, 46], and the interactions of enteropathogens such as *Salmonella enterica* [47] and *Cryptosporidium parvum* with the intestinal epithelium [48], here we have microinjected for the first time microbes in the lumen of intestinal organoids, imaged in real time microbial crossing of the intestinal barrier, and deciphered the underlying molecular mechanisms of microbial translocation. This novel *ex vivo* system of infection opens a wide range of opportunities to study pathogen interactions with host barriers in a direct manner, in contrast to what has been done so far. Additionally, this study also pioneers the use of genetically modified inducible organoids to address the cellular and molecular mechanisms of host-pathogen interactions in a tissue context. Rab11 has been shown to be necessary for Ecad recycling in mammalian cells and *in vivo* in *Drosophila melanogaster* [8]. Moreover, Rab11 is required for basolateral trafficking of newly synthesized Ecad in polarized MDCK cells [38]. However, the cellular trafficking of Ecad from its apical to basolateral pole from either side of *adherens* junctions had not been thoroughly investigated in a tissue context to our knowledge. It has been previously shown in non-polarized epithelial intestinal Caco-2 and trophoblastic JEG3 cell lines that, upon binding of InlA to Ecad, the plasma membrane is remodeled in an actin-dependent manner and leads

279 to caveolin- and clathrin-dependent bacterial endocytosis [32, 49-53]. In cultured epithelial
280 cells, *Listeria* internalization occurs mainly at the edge of cell islets, where cells are non-
281 polarized and Ecad is broadly accessible. Once internalized into these non-polarized cells, *Lm*
282 escapes from the vacuole in an LLO dependent manner and propels itself through ActA in the
283 cytosol [54]. Here, we have shown that in polarized differentiated goblet cells in a tissue
284 context, the initial step of internalization through accessible Ecad is also dynamin-dependent.
285 However, after completing bacterial internalization, *Lm* is rapidly transferred and released to
286 the basolateral pole of goblet cells, owing to the rapid Rab11-dependent recycling of Ecad.
287 This may protect invading bacteria from epithelial innate immune responses and favor their
288 dissemination in host tissues. Live imaging of host-pathogen interactions in organoids is a
289 promising approach to dissect their underlying cell and molecular biology.

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Authors' contributions

M. Lecuit conceived the project, designed the study, directed the research and edited the manuscript. M.K. designed and performed experiments, analyzed data and wrote the manuscript. C.F. designed and performed some experiments and analyzed some of the data. M. Lavina provided technical help. O.D. participated in the supervision of the study, data analysis and writing & editing of the manuscript.

Declaration of interests

The authors declare that there is no competing financial interest in this study.

Figure 1. *Ex vivo* intestinal organoid model of *Listeria* infection

(A) Experimental scheme and example of *Listeria* microinjection (details in the Methods). (B) Example images of *Listeria* translocation (optical section). *Lm* within a goblet cell, fixed 1 hour post microinjection (left panel; goblet cell marked with an arrow). *Lm* basolaterally extracellular beneath a goblet cell (right panel; goblet cells are marked with arrows). See also Video S1. Translocated bacteria were distinguished from the total bacteria by consecutive immunostaining, first against *Lm* without permeabilization followed by tissue permeabilization and labeling (Method). Scale bars, 10 μ m (C) Quantification of *Listeria* translocation. Top: Number of cells associated with bacteria either intracellularly or basolaterally extracellularly per organoid. Bottom: Number of translocated bacteria per organoid. Counts were performed in 12 organoids for each condition. Two-Way ANOVA test. NS: Not significant; ****: $p < 0.0001$. See also Figure S1D,E, Video S1. (D) Light sheet live imaging at indicated time point (top, maximum intensity projection (MIP); bottom, optical section). Within 12 minutes, bacteria-containing vesicle reaches the basolateral side of the cells, then exit from the cell in the following time points. Intracellular *Listeria* is surrounded with membrane tomato (arrow). See also Video S2. (E) Left: Scheme depicting detection of lumenally accessible Ecad from side and top view. Organoids were fixed, embedded, sectioned to open the lumen and immunolabeled from the luminal side without permeabilization. Ecad in the sectioned plane is exposed, thus accessible without permeabilization and stained throughout the cutting plane (marked with green on the drawing). Right: 3D-view of opened organoids from the side (top left) and top (bottom left). Enlargement of boxed area on the left 3D-reconstructed organoids containing goblet cells expressing lumenally accessible Ecad (center & right). Mucus that has been expelled is marked with WGA conjugated with fluorophore. Accessible Ecad surrounds the expelled mucus. Scale bars, 10 μ m. See also Video S3. (F) Left: Scheme depicting detection Ecad from side and top view. Organoids were fixed, embedded, sectioned to open the lumen, permeabilized and immunolabeled from the luminal side. Total Ecad is stained (marked with green on the drawing). Right: 3D-view of opened organoids from the side (top left) and top (bottom left). Enlargement of boxed area on the left 3D-reconstructed organoids containing goblet cell (center & right). Scale bars, 10 μ m. See also Video S3.

Figure 2. Endocytosis and microtubule dynamics are required for bacterial translocation

(A) Top: Experimental scheme of microinjection. Bottom: Confocal imaging of permeabilized, whole-mount stained organoids treated with indicated inhibitors. In cholchicine treated organoids, epithelium displayed more metaphase-blocked cells (+, enlarged), indicating that cholchicine treatment worked (B) Quantification of number of cells associated with bacteria. (C) Quantification of number of bacteria per organoid in indicated location. Experiments were repeated 2 to 3 times. The results combine all experiments. 27 WT & dynasore-treated organoids and 21 colchicine-treated organoids were counted. Kruskall Wallis test. Comparison to WT. NS: Not significant; *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$. See Also Figure S3. (D) Accessible Ecad from the luminal side of the sectioned organoids treated with indicated inhibitors. See also Figure S2, Video S4. (E) Confocal images of bacteria trapped inside the goblet cells in each condition (optical section from 3D reconstruction, goblet cells are marked with an arrow). Bacteria are located in apical and central side within the cells in dynasore- and cholchicine-treated conditions, respectively. Bacteria are surrounded with a tomato-stained membrane. See also Figure S3 and Video S4.

Figure 3. Inducible organoids expressing dominant-negative Rab11 and Rab7

(A) Scheme depicting the organoid transduction. (B) Visualization of successful induction with Dox for 16 hours. mCerulean (mCer) expression was detected by eyes using Zeiss Filter set 38 HE (excitation BP 470/40, beamsplitter FT 495, emission BP 525/50). (C) Confocal imaging of permeabilized, whole-mount stained organoids induced with indicated DN proteins for 16 hours with Dox. Expression of DN proteins is visualized by anti-Rab7 (Rab7DN) or anti-myc (Rab11DN) staining. In mycRab11DN-induced organoids, mCer co-localized with myc staining (not shown). Goblet cell producing mucus are labeled with WGA (arrow). Junctional integrity of organoids is evaluated by Ecad staining.

Figure 4. *Listeria* translocation requires functional Rab11 but not Rab7

(A) Experimental scheme of microinjection. The organoids were grown in presence of antibiotics (penicillin-streptomycin, geneticin and puromycin) and doxycycline was added 12-16 hours before the microinjection. Induction of mCerulean was detected by eyes with Zeiss Filter set 38 HE (excitation BP 470/40, beamsplitter FT 495, emission BP 525/50, Figure 3B). Induced organoids were selected and microinjected, incubated for 2 hours (DN proteins still being induced) followed by fixation & imaging. (B) Luminally accessible E-cadherin of the sectioned organoids induced with indicated DN proteins. Accessible E-cadherin staining in XY, XZ and YZ planes from indicated goblet cells (arrow from the right 3D reconstruction, sectioned plane facing up in the side view). See also Video S5. (C) Optical sections from 3D reconstruction of microinjected organoids induced for indicated DN proteins. *Listeria*-GFP (InlA) can be detected beneath the basolateral side of the goblet cells in transduced, but non-induced control (top) and in Rab7DN-induced organoids (center), indicating that bacterial translocation occurred. *Listeria*-GFP (InlA) is trapped at the basal pole of the goblet cell, embedded in WGA⁺ material in mycRab11DN-induced organoids (bottom). Goblet cells are marked with an arrow. Scale bars, 20 μ m. See also Figure S3 and Video S5. (D) Quantification of number of cells associated with bacteria per organoids. Kruskal Wallis test. Comparison to WT. NI: Lentivirus transduced, non-induced control. NS: not significant. (E) Quantification of number of bacteria per organoid in indicated location. Experiments were repeated 2 to 3 times. The results combine all experiments. Counts were performed in 19 non-induced, 18 Rab7DN and 24 Rab11DN organoids. The results for WT organoids are those from Figures 2B,C. Kruskal Wallis test. Comparison to WT. NS: Not significant; *: $p < 0.05$; **: $p < 0.01$. See also Figure S3.

STAR Methods

RESOURCES AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marc Lecuit (marc.lecuit@pasteur.fr).

Materials Availability

All plasmids generated in this study are available upon request.

Data and Code Availability

This study did not generate/analyze datasets or codes

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacteria

For Figure 1B-C, *Lm* strain EGD (BUG 600) and isogenic deletion mutants $\Delta inlA$ (BUG 947 [56]), Δhly (BUG 2132 [57]), $\Delta actA$ (BUG 2140 [57]), *Li* WT (BUG 499), *Li*-expressing InlA (*Li* (InlA+), BUG 1489 [23]) were used. We generated *Li*-expressing both InlA and GFP in tandem under the phyper promoter of pAD vector, separated by the terminator sequence (MBHL 366). Bacteria were transformed by electroporation. GFP expression was confirmed by fluorescent microscopy, function of InlA was confirmed by *in vitro* invasion assay using mouse fibroblast L2071 expressing human E-cadherin[49].

Mice

E16P KI mice were generated in the laboratory [16]. They were crossed with mT/mG mice [59] to generate mtd-Tmt; E16P KI mice. Animal experiments were performed according to the Institut Pasteur guidelines for laboratory animals' husbandry and in compliance with European regulation 2010/63 EU. They were approved by the ethical committee CETEA/CEEA No. 89 of Institut Pasteur under the number DHA180011.

Organoids

Intestinal organoids were generated and cultured from the crypts recovered from small intestines of 6- to 8-week old KIE16P and mtd-Tmt; E16P KI mice using EDTA dissociation method [9, 60]. They were grown in ENR medium (Advanced DMEM/F12 with EGF (50 ng/ml), Noggin (100 ng/ml) and R-spondin1 (500 ng/ml)).

Cells

HEK293T cells (ATCC CRL-11268) were grown and passaged in DMEM containing 2% glutamine, 10% Tet-system approved FBS (Takara # 631106) and penicillin-streptomycin.

METHOD DETAILS

Organoid microinjection

Mature organoids growing in ENR medium were mechanically dissociated and passed into the gridded, low-bottom iBidi-injection plate (Clinisciences # 80156). Result of Figure 1B-C was obtained with ENR medium while all others were obtained using the following condition: Organoids were passed in injection plates with 2:1 ratio of ENR and 50% of L-WRN cell-conditioned media (made with Tet System Approved FBS, Takara # 631106) for 1-2 days to have a round center to facilitate microinjection. When the organoids produced spherical centers, the organoids were washed several times with pre-warmed Advanced DMEM/F12 to remove the serum and excessive growth factors, replaced with either ENR or 5% L-WRN conditioned media for minimal 1 day. A day before the microinjection, 10 μ M DAPT and 20 μ g/ml IL-13 were supplemented overnight to partially enrich the goblet cells and to induce mucus expel from the existing goblet cells [61-63]. Figure 2A, final concentration of 80 μ M dynasore or 10 μ g/ml colchicine were applied 2 hours prior to microinjection. Figure 4A & C, final concentration of 2 μ g/ml doxycycline was added to the media 12-16 hours before the microinjection. Microinjection was performed only to organoids verified to have successful induction. Results of Figure 1B-C were obtained by fixing 1 hour and 16 hours, respectively, after microinjection. The incubation period post microinjection of the other experiments was optimized to 2 hours followed by fixation to reduce the damage of the organoids. Drug/doxycycline treatment was maintained during this period. For live imaging, immediately after microinjection, organoids were re-sampled to the imaging chamber of light sheet microscope Zeiss Z1.

Overnight culture of bacteria was diluted (1/200) and grown until O.D 0.8, washed at least 3 times in PBS and collected as a final volume of 100 μ l in DMEM/F12 phenol red-free

medium. Microinjection was performed with Eppendorf InjectMan and FemtoJet system as reported [64] using glass micropipettes injection needle (Vitromed # V-INJ-S3-35).

Accessible Ecad detection

Organoids were passed on the 8-well Lab-Tek plates and supplied with the media as for the microinjection: first with 2:1 ratio of ENR and 50% L-WRN cell conditioned media to grow the spherical center, followed by washing and either returned to ENR media or supplied with 5% L-WRN media for at least 1 day. A day before the fixation, organoids were treated at a final concentration of 10 μ M DAPT and 20 ng/ml IL-13 overnight to induce mucus expel from the existing goblet cells. Organoids were fixed with 4% PFA at 4°C overnight. For the experiments in Figure 2D, final concentration of 80 μ M dynasore or 10 μ g/ml colchicine were added 2 hours before fixation. Experiment of Figure 4B, final concentration of 2 μ g/ml doxycycline was added 12-16 hours before fixation. When the organoids were fixed, PFA was removed, organoids were washed and 6% low-melting agarose was poured to the wells. Solidified wells were recovered, sectioned in 150-200 μ m thickness with a vibratome (Micro HM 650V, Thermo Fisher Scientific). Sections containing opened organoids were blocked in 3% BSA in PBS and stained as indicated below.

Immunofluorescence

Accessible Ecad (Figure 1E, 2C and 4B): Organoids were fixed with 4% PFA at 4°C overnight followed by washing and blocking with 3% BSA in PBS, overnight at room temperature. Primary and secondary antibodies were applied for 1 hour at room temperature.

Whole-mount staining: Organoids were fixed with 4% PFA for 30 min – 1 hour at room temperature followed by washing, blocking/permeabilizing for 2 hours either in 5% goat serum with 1% Triton X-100 or in 3% BSA with 1% Triton X-100 in PBS. Primary antibodies were applied at 4°C overnight followed by washing and stained with secondary antibodies for 2 hours at room temperature or 4°C overnight.

Distinguishing intracellular vs translocated bacteria (Figure 1B): Organoids were fixed with 4% PFA at 37°C for 1 hour, washed and blocked without triton for 2 hours at room temperature. Rabbit antisera against *Listeria monocytogenes* (R11 [55]) was added for 2 hours at room temperature, followed by washing and secondary antibody for 1 hour at room temperature to stain extra-organoid bacteria. Then microinjected organoids were

permeabilized with 1% Triton X-100 and stained with primary antibody at 4 °C overnight followed by washing and secondary antibody for 2 hours at room temperature.

The following antibodies were used: anti-Ecad (Ecc2, Takara #M108, 1:350), anti-myc (71D10, Cell Signaling # 2278, 1:500), anti-Rab7 (Sigma, #R4779, 1:200), WGA conjugated with alexa 647 (Invitrogen, whole mount 1:300, section 1:1000) and Hoechst 33342 (Invitrogen 1:5000). Secondary antibodies include goat anti-rat conjugated with alexa 546, goat anti-mouse conjugated with alexa 405, and goat anti-rabbit conjugated with alexa 405 (all Invitrogen 1:500). To identify the goblet cells, the following criteria were used, as the Muc2 labeling method following Carnoy fixation and paraffin embedding [65] is not compatible with our system, rendering the matrigel opaque as well as breaking the plate: WGA⁺, typical goblet-shaped cellular morphology including the opening of the apical area and the nucleus misaligned with neighboring cells. If the cells meet 2 or more conditions, we regarded them as goblet cells.

Imaging

Images were acquired either by confocal microscope (fixed image, upright Zeiss LSM 700 equipped with a water Plan-Apochromat 40x/1.0 DIC M27 objective & inverted Zeiss LSM 710 equipped with an oil Plan-Apochromat 40x/1.3 DIC M27 objective) or light sheet microscope (live image, Zeiss Z.1 equipped with a water Plan-Apochromat 40x/1.0 DIC objective). For live imaging, imaging chamber was maintained at 37°C temperature, 5% CO₂ and supplemented with ENR media made with phenol red-free DMEM/F12. Three-dimension reconstruction was performed using Arivis Vision 4D. 3.0.1 software. For Figure 1D and video S2, the image was denoised using median filter (radius 1) followed by background correction.

Rab11DN and Rab7DN vector construction

Rab11DN and Rab7DN were generated by mutagenesis PCR from the pCMV-intron myc Rab11WT (Addgene #46785) and pCMV-SPORT6-Rab7, respectively.

Primers used for Rab11DN are: 5'-GTGTTGGAAAGAACAACCTCCTGTCTCGATTTA-3' & 5'-GACAGGAGGTTGTTCTTTCCAACACCAGAATC-3'. Primers used for Rab7DN are: 5'-CTGGTGTGGAAAGAACTCTCTCATGAACCAG-3' & 5'-CTGGTTCATGAGAGAGTTCTTTCCAACACCAG-3'. DN constructs were cloned into the multiple cloning site I (MCSI) and mCerulean sequence was cloned into the MCS II of Tet-

On® 3G Inducible Expression System (Bicistronic Version, pTRE3G-IRES, Takara # 631166)

Lentivirus production and concentration

Lentivirus particle containing tet-activator was purchased from Takara (#631311). Lentivirus containing inducible mycRab11DN-mCer and Rab7DN-mCer constructs were produced using calcium phosphate transfection in HEK293T cells (Takara # 631312, user protocol). 2nd generation lentiviral plasmids psPAX2 and pMD2.G were used (Addgene #12260, #12259). Lentivirus was concentrated with PEG-itTM (System Bioscience, # LV810A-1, user protocol).

Organoid transduction

Organoids were transduced with lentivirus using the method modified from Maru *et al.*, 2016 and Van Lidth de Jeude *et al.*, 2015 [11, 12]. Briefly, 1st generation of organoids from the crypts were passed with ENR medium containing Wnt3a, CHIR99021 and Nicotinamide to enrich stem cell population. 2-3 days later when the organoids display spheroid morphology with few dead cells in the lumen, organoids were mechanically broken down and trypsinized to single cells. Cells were washed and supplemented with the Lentivirus concentrate in a final concentration of 1X ENR media containing Wnt 3a, CHIR99021, Nicotinamide, TransDuxMAXTM with enhancer. Cells and virus mixture were incubated at 37°C water bath for around 1 hour, distributed to a matrigel-coated 24 well plates, incubated in the 37°C incubator for overnight. The next day, as live cells settle on the coated matrigel, supernatant containing virus and dead cell debris was carefully removed and fresh matrigel was added to form a sandwich having the live cells in the center of the two layers of the matrigel. ENR medium containing Wnt3a, CHIR99021 and Nicotinamide (without TransDuxMAXTM) was supplied for 2 more days. A final concentration of 1 µg/ml puromycin and 100 µg/ml geneticin (neomycin) were added to select for the successful transduction with pTRE3G-DN mutant-IRES;mCerulean and tet-activator, respectively, until the transduced stem cells form small visible organoids. Media was returned to regular ENR media containing three antibiotics (pen-strep, puromycin and geneticin) and transduced organoids were cultured in presence of the three antibiotics throughout.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

555 All statistical analysis has been performed using Prism 8 (Graphpad). Details for statistical
556 tests used can be found in figure legends, including the number of replicates performed and
557 number of organoids analyzed for each condition and p-value

558

Video legends

Video S1. *Listeria* crossing through intestinal organoid goblet cells. Related to Figure 1B and Figure S1D. 3D reconstruction of an intraluminally microinjected organoid, fixed 1 hour post injection. *Lm* (time 00:00-00:15: green, endogenous GFP thus intracellular; time 00:16-00:25: cyan, double-labelled, thus translocated, see methods), Ecad (white) and WGA (red) are indicated. 3D reconstruction (time 00:26-00:43, time 01:12-01:25) and optical section (time 00:44-01:11) of *Li*-GFP (InlA) intraluminally microinjected organoids, expressing membrane tomato and fixed 2 hours post injection. WGA (white) and nucleus (blue) are indicated.

Video S2. Real-time imaging of *Listeria* translocation. Related to Figure 1D.

Intraluminally microinjected organoids expressing membrane tomato were imaged every 6 minutes for 2 hours. First and last horizontal rotation shows initial and last time point (00:00:00 and 01:30:00), respectively. Bacterial translocation initiated between time point 8 and 9 (00:42:00 and 00:48:00) and ended between time point 15 and 16 (01:24:00 and 01:30:00). The white arrow indicates the bacterium being transcytosed.

Video S3. Mucus-expelling goblet cells express lumenally accessible Ecad. Related to Figure 1E, F. Organoids were fixed, embedded in the agar, sectioned, immunolabeled from the lumen without permeabilization (time 00:00-00:12) and with permeabilization (time 00:13-00:25). Ecad (green), WGA (red) and nucleus (blue) are indicated. Note that Ecad in the sectioned plane is exposed, thus accessible without permeabilization and stained throughout the cut plane. Goblet cell enlarged in Figure 1E is marked with an arrow.

Video S4. *Listeria* translocation is impaired when Ecad endocytosis and microtubule dynamics are inhibited. Related to Figure 2D, E. Lumenally accessible Ecad in mucus-expelling goblet cells in dynasore-treated organoids (time 00:00-00:12) and in cholchicine-treated organoids (time 00:13-00:24). Apical intracellular *Listeria* in dynasore-treated organoid (time 00:25-00:36) and *Listeria* in the central part inside the goblet cell in cholchicine-treated organoids (time 00:37-00:48).

Video S5. *Listeria* translocation is impaired in Rab11DN but not in Rab7DN-induced organoids. Related to Figure 4B, C. Lumenally accessible Ecad in mucus-expelling goblet cells in Rab7DN-induced organoids (time 00:00-00:12) and in Rab11DN-induced organoids

593 (time 00:13-00:24). *Listeria* translocated across the goblet cells in Rab7DN-induced organoid
594 (time 00:25-00:44) and *Listeria* trapped in the basolateral pole of goblet cell in Rab11DN-
595 induced organoids (time 00:45-01:01). *Listeria* trapped in mucus inside the goblet cell is
596 indicated by an arrow.
597 .

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies and fluorescent dyes		
Rat anti-E-cad (Ecc2)	Takara	#M108
Rabbit anti Rab7	Sigma	R4779
Rabbit antisera against <i>Listeria monocytogenes</i>	[55]	R11
Mouse IgG1 anti-myc (71D10)	Cell signaling	#2278
WGA-conjugated with Alexa Fluor 647	Invitrogen	W32466
Hoechst 33342	Invitrogen	H3570
Goat anti-Rat Alexa Fluor 546	Invitrogen	#A-11081
Goat anti-Rabbit Alexa Fluor 405	Invitrogen	#A-31556
Goat anti-Mouse Alexa Fluor 405	Invitrogen	#A-31553
Bacterial and Virus Strains		
<i>Lm</i> strain EGD	[56]	BUG600
EGD Δ <i>inlA</i>	[56]	BUG 947
EGD Δ <i>hly</i>	[57]	BUG 2132
EGD Δ <i>actA</i>	[57]	BUG 2140
<i>Li</i> WT	[55]	BUG 499
<i>Li</i> (InlA+)	[23]	BUG 1489
<i>Li</i> -GFP (InlA+)	This paper	MBHL 366
rLV.EF1.Tet3G-9	Takara	631311
Chemicals, Peptides, and Recombinant Proteins		
N2 Supplement	Gibco Invitrogen	#17502048
B27 Supplement	Gibco Invitrogen	#17504044
N-Acetylcystein	Sigma Aldrich	#A9165-5G
Human recombinant R-spondin 1 (final 500 ng/ml)	R&D systems	#4645-RS250
Mouse recombinant Noggin (final 100 ng/ml)	Peprtech	#250-38-20ug
Mouse recombinant EGF (final 50 ng/ml)	Invitrogen	#PMG8044
Y-27632 (final 10 uM)	Sigma Aldrich	#Y0503-1MG
Mouse recombinant Wnt3a (final 100 ng/ml)	Millipore	#GF160
Nicotinamide (final 10 mM)	Sigma Aldrich	#N0636-100g
CHIR99021 (final 10 uM)	Stemgent	#248040004
Doxycycline (final 2 ug/ml)	Takara	#631311
Dynasore (final 80 uM)	Sigma	324410-10MG
Colchicine (final 10 ug/ml)	Sigma	C3915
DATP (final 10 uM)	Stemgent	#04-0041
IL-13 (final 20 ng/ml)	R&D systems	#413-ML-005
TransDux MAX TM	System Bioscience	LV860A-1
Matrigel	Corning	356231
Cell recovery solution	BD	354253
Advanced DMEM/F12	Gibco Invitrogen	12634010
DMEM/F12 phenol red-free	Gibco Invitrogen	21041025
GlutaMAX	Gibco Invitrogen	35050038
1M Hepes	Gibco Invitrogen	15630056
Penicillin-streptomycin	Gibco Invitrogen	15140163
Puromycin (final 1 ug/ml)	Sigma	540411-25MG

Neomycin (Geneticin, final 1X)	Gibco Invitrogen	10138031
Critical Commercial Assays		
Calcium phosphate transfection kit	Takara	#631312
PEG-it TM Virus precipitation solution	System Bioscience	# LV810A-1
Experimental Models: Cell Lines		
HEK293T	ATCC	#CRL-11268
L-WRN	ATCC	#CRL-3276
Experimental Models: Organisms/Strains		
Mouse (KI E16P) intestinal organoid	This paper and [16]	N/A
Mouse (mtd-Tmt; KI E16P) intestinal organoid	This paper and [6]	N/A
Oligonucleotides		
Primer for Rab11DN 5'-GTGTTGGAAAGAACAACCTCCTGTCTCGATTTA-3'	This paper	N/A
Primer for Rab11DN 5'-GACAGGAGGTTGTTCTTTCCAACACCAGAA TC-3'	This paper	N/A
Primer for Rab7DN 5'-CTGGTGTGGAAAGA ACTCTCTCATGAACCA G -3'	This paper	N/A
Primer for Rab7DN 5'-CTGGTTCATGAGAGAGTTCTTTCCAACACCAG -3'	This paper	N/A
Recombinant DNA		
pAD	[58]	N/A
psPAX2	Addgene	#12260
pMD2.G	Addgene	#12259
pTREG3-IRES	Takara	#631312
pCMV-intron-mycRab11	Addgene	#46785
pCMV-SPORT6-Rab7	Addgene and this paper	N/A
Software and Algorithms		
Prism 8	Graphpad	
Arivis Vision4D 3.0.	Arivis	
FIJI	ImageJ	
ZEN 2014 SP1	ZEISS

A **B** **C**

D Number of cells associated with bacteria / organoid

E Number of bacteria / organoid

Figure S1. Expression of InlA by *Listeria innocua* allows translocation across the intestinal organoid epithelium. Related to Figure 1.

(A) Cell subtype heterogeneity in organoids. Enterocytes (villin⁺), enteroendocrine cells (chromograninA⁺), goblet cells (WGA⁺/Lysozyme^{low}), Paneth cells (Lysozyme⁺/WGA^{low}) and M cells (GP2⁺) are shown. Note that M cells are absent in intestinal organoids. (B) Goblet cell (arrow) labeled with WGA and cytokeratin-18. Besides the labeling, note that goblet cell displays cup-shaped morphology with apical side opened and/or squeezed nucleus. (C) % of WGA⁺ cell population in *Listeria* non-injected (left, control) and microinjected (right) organoids. Organoids were incubated for 16 hours post microinjection, non-injected control is from the same plate (non-injected neighboring organoids). Two-Way ANOVA test. NS: Not significant; p=0.61. (D) Confocal images of *Li*-GFP(InlA) (arrows) underneath the goblet cells after microinjection. 3D reconstruction and optical sections of boxed area are shown. Mucus material is not visible in the organoids on the right due to the fixation, thus goblet cells (asterisk) are identified based on typical goblet-shaped cellular morphology with opening of its apex. (E) Left: 3D reconstruction of an organoid showing translocated bacteria together with WGA⁺ stained material, indicating that the bacteria exit the goblet cell with the mucus. Center & Right: Optical section of the boxed area on the left. Mucus labeled with WGA that exited along with the bacteria is marked with an arrow.

Figure S2. Inhibition of Ecad recycling pathway impairs *Lm* transcytosis. Related to Figure 2, 3 and 4.

(A) % of goblet cells in organoids of indicated settings. Expelled mucus was identified with WGA staining. Organoids were briefly treated with DAPT and IL-13 to partially enrich goblet cell and mucus secretion (Method). (B) Distribution of lumenally accessible Ecad on goblet cells in organoids with indicated treatments. (C) Ecad localization in indicated conditions. In control organoids, Ecad endocytic punctae can be seen in the cytosol (1st column arrow), which is abolished when Dynasore is added (2nd column). Ecad aggregates can be seen in Rab11DN-expressing organoids (3rd column), suggesting that Ecad recycling/release is perturbed. Ecad in Rab7DN-expressing organoids are observed in big cytosolic aggregates (box 1) or dispersed cytosolic punctae (box 2), indicating that Ecad degradation is perturbed. Scale bar, 20 μ m. (D) Organoids microinjected with WT *Listeria* were incubated for 16 hours in presence of doxycycline to induce DN protein. While *Listeria* translocates across the goblet cell in transduced, non-induced control (NI, left), *Listeria* is trapped at the basal pole inside the goblet cell in Rab11DN induced organoids (right). Goblet cells are marked with a star. Scale bar, 20 μ m. (E) Quantification of number of cells associated with bacteria per organoids. Mann-Whitney test. NS: not significant. (F) Quantification of number of bacteria per organoids in indicated location. Counts were performed in 10 non-induced and 21 Rab11DN organoids. Mann-Whitney test. NS: non significant; *: $p < 0.05$.

Difference with WT	Dynasore	Colchicine	Rab11DN	Rab7DN	Non induced
Apically-associated	**	NS	NS	NS	NS
Intracellular Central	NS	****	NS	NS	NS
Intracellular Basolateral	NS	NS	****	NS	NS
Extracellular Translocated	*	**	*	NS	NS

Figure S3. Molecular mechanism involved in *Listeria* transcytosis across the goblet cell.

Related to Figure 2 and 4.

Top: Graphical illustration combining the results of microinjection, with the total number of bacteria counted in each condition of organoids. Rab7DN-induced and transduced but non-induced organoids display basolaterally extracellular bacteria as WT organoids. This indicates that bacteria translocate across the organoid epithelium independently of Rab7-mediated Ecad degradation. Inhibition of dynamin-mediated endocytosis in organoids by dynasore leads to significantly increased number of bacteria that are apically associated to the goblet cells, but significant decrease in the number of translocated bacteria. This indicates that Ecad endocytosis is involved in the step of bacterial entry. Microtubule dynamics inhibition by colchicine also leads to significant decrease of translocated bacteria counts, but significant increase of intracellular bacteria that are located near the center of the cells. This indicates that bacteria-containing vesicles transit *via* microtubule within the goblet cells. Rab11DN-induced organoids show significant increase in the number of intracellular bacteria located in the basolateral area of goblet cells, but significant decrease in the number of bacteria elsewhere, suggesting that functional Rab11 is required in completing bacterial translocation. **Bottom:** Table summarizing the statistical significances. Kruskal Wallis test. Comparison to WT. NS: Not significant; *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$