TLR sorting by Rab11 endosomes maintains intestinal epithelial-microbial homeostasis

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**Abstract**

Compartmentalization of Toll-like receptors (TLRs) in intestinal epithelial cells (IECs) regulates distinct immune responses to microbes; however, the specific cellular machinery that controls this mechanism has not been fully identified. Here we provide genetic evidences that the recycling endosomal compartment in enterocytes maintains a homeostatic TLR9 intracellular distribution, supporting mucosal tolerance to normal microbiota. Genetic ablation of a recycling endosome resident small GTPase, Rab11a, a gene adjacent to a Crohn’s disease risk locus, in mouse IECs and in Drosophila midgut caused epithelial cell-intrinsic cytokine production, inflammatory bowel phenotype, and early mortality. Unlike wild-type controls, germ-free Rab11a-deficient mouse intestines failed to tolerate the intraluminal stimulation of microbial agonist, thus, Rab11a endosome controls intestinal host-microbial homeostasis at least partially via sorting TLRs.

**Keywords** enterocyte; inflammation; intestinal homeostasis; Rab11a; Toll-like receptor

**Subject Categories** Cell Adhesion, Polarity & Cytoskeleton; Immunology

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**Introduction**

A finely tuned immuno-surveillance system that exquisitely balances immuno-responsive and immuno-repressive activities is necessary for microbe–host homeostasis in animal tissues. Genetic and environmental factors that disrupt this balance may underlie various immunological disorders including the inflammatory bowel diseases (IBDs). In mammals, the postnatal intestinal epithelial cells (IECs), after transitioning from a relatively germ-free fetal environment, immediately interact with enteric microbes and participate in immune surveillance against luminal pathogenic stimuli (Artis, 2008; Maynard et al., 2012). Mature human IECs appear to use specific pathogen pattern recognition receptors such as the Toll-like receptors (TLRs) to balance immune tolerance and immune response, depending on specific cellular localization of the receptors (Abreu, 2010). In cultured human colon epithelial cells, bacterial cytosine-guanine (CpG) stimulation of apically localized TLR9 from the luminal side induced tolerance to subsequent microbial agonist stimulations, whereas basolateral stimulation of TLR9 provoked NF-xB activation and cytokine production (Lee et al., 2006). In contrast, the exclusive basolateral localization of TLR5 in IECs appeared to facilitate this sensor to only respond to the invaded bacterial flagellin protein after barrier function impairment (Gewirtz et al., 2001; Rhee et al., 2005). Furthermore, apical localization of TLR4 was described in human colon epithelial cell lines, and this receptor changed its subcellular localization upon ligand stimulation (Cario et al., 2002). In the small intestine, TLR4 signaled from endosomes in response to its internalized ligand lipopolysaccharide (LPS), allowing differentiation between various types of LPS (Hornef et al., 2003). In addition to receptor compartmentalization, proteolytic cleavage of TLR9 in endolysosomal compartment provides another crucial control for proper receptor activation in immune cells (Ewald et al., 2008; Park et al., 2008). However, the absolute requirement of this proteolytic processing and the role of cleaved N-terminal domain for TLR9 activation are still under intensive studies (Peter et al., 2009; Mouchess et al., 2011; Onji et al., 2013).

Since adverse activation of microbial sensors could elicit unwanted immune responses driving intestinal pathogenesis (Leaphart et al., 2007; Fukata et al., 2011), there is a clear imperative to understand the IEC-intrinsic sorting units controlling the proper compartmentalization and activation of TLRs. A recently reported Crohn’s disease risk locus at chromosome 15q22 (rs17293632) is adjacent to the human RAB11A (Franke et al., 2010). This gene encodes a small GTPase representing one of the most prominent components of a special endosomal subpopulation—the recycling endosome (Goldenring, 2013). Studies in cultured cell

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lines suggested that the Rab11A endosome engages in intense membrane recycling and sorting, and connect the endo- and exocytic pathways (van Ijzendoorn, 2006). In cultured human colonic epithelial cells, Rab11A deletion caused abnormal lumen formation. In mouse intestines, Rab11a expression is increased in IECs during cellular differentiation and maturation (Gao & Kaestner, 2010). Here, we used genetic and biochemical approaches to show that Rab11a endosomal compartment maintains homeostatic TLR9 compartmentalization at steady-state conditions. By doing so, Rab11a appeared to prevent unwanted pro-inflammatory stimuli. Genetic and cell type-specific inactivation of Rab11a in mouse and Drosophila IECs midgit caused aberrant NF-kB activation, inflammatory cytokine production, and IBD phenotypes. Removal of microbial ligands (germ-free) alleviated these phenotypes in the mutants. Unlike wild-type controls, germ-free Rab11a<sup>IEC</sup> mice failed to tolerate intraluminal perfusion of microbial TLR agonists. Our data suggested that Rab11a controls intestinal microbial tolerance at least partially via sorting TLRs.

**Results**

**Rab11a ablation in intestinal epithelia causes inflammation**

To study the contribution of Rab11a recycling endosome to intestinal host-microbial homeostasis, we derived a Rab11a floxed (fl) conditional mouse allele. Rab11a global knockout mice (Rab11a<sup>−/−</sup>) died in utero around the implantation stage (data not shown); therefore, we established IEC-specific Rab11a knockout mice (Rab11a<sup>fl/fl; Villin-Cre<sup>+</sup></sup>, or Rab11a<sup>IEC</sup>) using the Villin-Cre transgene. Rab11a<sup>IEC</sup> mice were born at the expected Mendelian ratio; however, at a young age, they exhibited significant runting (Fig 1A and B, see asterisk), and an approximately 40% mortality rate at the age of weaning. Male mutant mice showed higher mortality rate than their female counterparts; survivors also displayed higher mortality with aging (data not shown). Both male and female mutant mice had a dilated intestinal lumen and a shortened colon (Fig 1A, see asterisk), and an approximately 40% mortality rate at the age of weaning. Male mutant mice showed higher mortality rate than their female counterparts; survivors also displayed higher mortality with aging (data not shown). Both male and female mutant mice had a dilated intestinal lumen and a shortened colon compared with their wild-type littermates (Fig 1C, see asterisks). Western blot confirmed the removal of Rab11a protein from Rab11a<sup>IEC</sup> mouse intestinal epithelia (Fig 1D). In contrast to control intestinal epithelia, where Rab11a was detected by immunohistochemistry in the subapical cytoplasm of villus epithelial cells (top panels, Fig 1E), Rab11a staining was absent from the Rab11a<sup>IEC</sup> intestinal villus epithelia (bottom panels, Fig 1E). Histopathologically, Rab11a<sup>IEC</sup> intestines showed blunted villi, reduced goblet cells, and macrophage infiltration into the submucosa (Fig 1F). In neonatal Rab11a<sup>IEC</sup> mice, the intestinal villi were frequently observed to fuse and branch (see also Supplementary Fig S1A). Pulse-chase BrdU labeling or immunostaining for phosphorylated Histone H3 (pH3) detected increased numbers of cycling crypt cells in Rab11a<sup>IEC</sup> mice at all postnatal (P) stages (Fig 1G). This crypt hyperplasia was consistent with the generally enlarged crypt morphology in these mutants (Fig 1G).

Increased crypt cell proliferation was also detected when Rab11a deletion was induced in adult Rab11a<sup>fl/fl; Villin-CreER<sup>+</sup></sup> mice by tamoxifen administration (Supplementary Fig S2), suggesting that the hyperproliferative response of crypt cells might not simply be a result of defective intestinal development. Indeed, the differentiation of enterocytes and three intestinal secretory cell lineages was unaffected in Rab11a<sup>IEC</sup> mice (enterocytes and goblet cells shown in Fig 1F, paneth and enteroendocrine cells in Supplementary Fig S3A). Rab11a-deficient enterocytes were still capable to elaborate apical brush borders (Supplementary Fig S3B). Therefore, Rab11a deletion did not appear to disrupt the differentiation of major intestinal cell lineages. However, the epithelial hyperplasia, dysplasia, and immune cell infiltration continued to be present in older survivor Rab11a<sup>IEC</sup> mice (5 months old shown in Supplementary Fig S1B), which showed high histopathology scores indicative of intestinal inflammation (Fig 1H) (Adolph et al., 2013). Finally, tamoxifen induced deletion of Rab11a specifically in Lgr5<sup>+</sup> intestinal epithelial stem cells (IESCs), or crypt-based columnar cells, CBCs in Rab11a<sup>fl/fl; Lgr5<sup>EGFP-IRESCreER</sup></sup> mice increased the proliferation of both the Lgr5<sup>+</sup> and the transit-amplifying cells (Fig 1I). No immediate cell death was triggered by inducible Rab11a deletions (data not shown).

To identify the mechanisms underlying the above pathological abnormalities, we performed microarray analysis on neonatal (postnatal day 3, P3) mouse intestines using four independent pairs of RNA samples from Rab11a<sup>IEC</sup> and wild-type littermates (Fig 2A). Inflammatory genes encoding cytokines, chemokines, defensins, and anti-microbial peptides were significantly upregulated as the most enriched gene category in Rab11a<sup>IEC</sup> intestines (Fig 2B and C). Overlapping analyses revealed that 79 and 138 upregulated transcripts in Rab11a<sup>IEC</sup> were shared by two independent mouse enteritis models: transgenic CD98<sup>IEC</sup> and trinitrobenzene sulfonate (TNBS)-induced enteritis, respectively (Nguyen et al., 2011; Avula et al., 2012) (Fig 2D). These findings from microarray analysis were confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) analyses, with IL6, CXCL1 (human IL8 homolog), and CXCL5 being the most robustly activated genes (Fig 2E). A number of genes downstream of canonical Wnt pathway were not changed. Activation of IL-6 signaling pathway in Rab11a<sup>IEC</sup> intestines was further supported by elevated levels of phosphorylated Stat3 (pStat3), a downstream effector of IL-6 (Fig 2F). Luminescent multiplex cytokine/chemokine assays identified elevated levels of IL-6, IL-1β, and MCP1 in Rab11a<sup>IEC</sup> mouse serum (Fig 2G), supporting an activated systemic inflammatory response in these mice.

Professional immune cells and IECs are both capable of producing inflammatory cytokines including IL-6 (Kusugami et al., 1995; Vinderola et al., 2005). To identify the sources of these cytokines, we first performed IL-6 immunofluorescent analyses. Higher IL-6 immunoreactivities were detected in Rab11a<sup>IEC</sup> intestinal epithelia compared with the control epithelia (Fig 2H). The strongest IL-6 signal in wild-type epithelia was found in immune cells resident in the lamina propria (arrowheads in Fig 2H). To determine whether Rab11a-deficient IECs were the origins of observed cytokine production, we performed intestinal organoid cultures and directly measured the secreted inflammatory cytokines within the supernatant of culture medium containing live organoids (Fig 2I). After 1 week in culture, Rab11a<sup>IEC</sup> organoids, presumably devoid of lymphocytes, produced higher amounts of IL-6, SDF-1α/CXCL12, and IL-1β measured by ELISA (Fig 2J). Of note, even in culture, Rab11a<sup>IEC</sup> organoids initiated bud outgrowths more rapidly and contained a larger population of proliferative cells than wild-type organoids (Fig 2I). Therefore, the isolated Rab11a<sup>IEC</sup> crypts might have contained more progenitor cells to start with. Deletion of Rab11a in cultured Rab11a<sup>fl/fl; Villin-CreER<sup>+</sup></sup> organoids through
administrating tamoxifen elicited a less potent increase of IL-6 secretion (Fig 2K), suggesting that loss of Rab11a triggered an epithelial cell-intrinsic cytokine production.

Rab11 depletion in Drosophila enterocytes caused IBD-like phenotype

The mouse Villin promoter-driven Rab11a deletion described above targeted all IEC cell types. It was not possible to distinguish the cell-autonomous mechanism from non-autonomous mechanism that was responsible for triggering the inflammatory responses. We employed an intestinal cell type-specific knockdown approach in Drosophila midgut—a tissue equivalent to mammalian intestine—using RNAi against Rab11, the only Rab11a homolog in fly genome. This system enabled us to inducibly delete Rab11 in a particular IEC cell type via tightly controlled Gal4 drivers: the Delta promoter-Gal4 (DlGal4) for IESCs, the Su(H) binding site-Gal4 [Su(H)Gal4] for enteroblasts (EBs), the escargot promoter-Gal4 (esgGal4) for IESCs and EBs, and the Myosin1A promoter-Gal4 (Myo1AGal4) specific for enterocytes (Micchelli & Perrimon, 2006; Zeng et al, 2010; Jiang et al, 2011) (Fig 3A). Entire midguts from cell-specific Rab11 RNAi and control RNAi adult flies were analyzed for pHH3+ mitotic cells. Remarkably, enterocyte-specific Rab11 knockdown, mediated by the Myo1AGal4 driver, caused the highest, nearly 100-fold, increase of mitosis in IESCs, whereas the esg and Su(H) drivers caused only modest increases of IESCs proliferation.
In addition to the Rab11TRiP RNAi line, two additional Rab11 RNAi lines (Rab11v22198 and Rab11v108382) targeting distinct Rab11 sequences led to similar phenotypes after crossing with the Myo1AGal4 driver (Fig 3C), suggesting the phenotype was Rab11-specific. Epithelial organization was determined by confocal microscopy based on Myo1A-driven GFP expressed only in enterocytes and the β-catenin (Armadillo) staining that highlighted the cell membrane (Fig 3D–I). In wild-type midgut, the IESC-EB cell nests normally contained 1–2 cells (Fig 3D, arrows). However, in Myo1AGal4-driven Rab11 RNAi midguts, the nests were composed of increased number of cells (Fig 3F and H), with the Rab11v108382 line showing the strongest phenotype with the highly expanded cell nests occupying most of the gut epithelium (Fig 3H). These cell nests were expanded from Rab11 wild-type IESC as they did not contain Myo1AGal4-driven GFP. Visualized from sagittal sections, these expanded cell nests continued to occupy the basal side of the...
epithelium (Fig 3E, G and I), illustrating an overall benign epithelial organization except for an increased precursor cell number due to IESC hyperproliferation. These results suggested that Rab11 depletion from enterocytes triggered neighboring IESC division, possibly through non-autonomous mechanisms.

Using qRT-PCR, we analyzed a variety of ligands and growth factors, including activators of the Wnt, JAK-STAT, and EGF signaling pathways (Jiang & Edgar, 2012) known to be critical for IESC proliferation in adult *Drosophila* midgut. Among these factors examined, Upd3, a *Drosophila* IL-6-equivalent ligand that activates the JAK-STAT pathway (Zhou et al. 2013), showed the highest increase (~600-fold) in its expression comparing to controls (Fig 3J). We used an Upd3 promoter-driven lacZ reporter line (Zhou et al. 2013) to determine the identity of cells within the midgut that contributed to this high level of Upd3 expression. LacZ expression was markedly increased in Rab11 RNAi midguts, with a pattern identical to enterocytes that were marked by Myo1A-driven GFP (Fig 3K). Collectively, the above results from mouse intestines and *Drosophila* midgut strongly suggested that Rab11-deficient enterocytes triggered cell-autonomous cytokine productions that indirectly impacted IESC proliferation.

Activation of NF-κB and MAPK pathways in *Rab11a<sup>−/−</sup>* intestines

To identify the inflammatory signaling pathways that were upstream of the epithelial cytokine responses in Rab11a-deficient intestines, we analyzed NF-κB, MAPK, and JNK pathways, known to be critically involved in inflammatory and stress responses (Pasparakis, 2009; Arthur & Ley, 2013). Western blots detected significantly elevated P65 and RelB protein levels in *Rab11a<sup>−/−</sup>* mice at all postnatal stages, but not at fetal stages (Fig 4A). Inducible deletion of *Rab11a* in adult *Rab11a<sup>−/−</sup>*; Vil-Cre<sup>ER</sup> mice by tamoxifen injection also increased the levels of P65 and RelB (Fig 4B). These changes in NF-κB protein levels likely reflected a specific response to the loss of Rab11a, as deletion of *Rab8a*, another small GTPase regulating apical membrane trafficking in IECs (Sato et al. 2007), did not increase P65 or RelB levels (Fig 4C). NF-κB activation in *Rab11a<sup>−/−</sup>* mouse intestines was also supported by elevated nuclear P65 levels from fractionated tissue lysates (Fig 4D). Abnormal activation of NF-κB was reported to cause intestinal inflammation (Zhang et al. 2006; Vereecke et al. 2010; Vlantis et al. 2011), similar to the phenotype of *Rab11a<sup>−/−</sup>* mice. Interestingly, we found that, during intestinal development in wild-type mice, P65 and RelB protein levels attenuated from late gestation stage. Rab11a levels increased during the time course, illustrating a reverse correlation (Fig 4E). Immunohistochemistry for P65 showed that postnatal *Rab11a<sup>−/−</sup>* intestinal villus epithelia, unlike wild-type littermates, continued to strongly express this master activator of inflammation (Ghosh & Hayden, 2008) (Fig 4F). In addition to NF-κB, activation of MAPK (Fig 4G) but not JNK pathway (data not shown) was detected in *Rab11a<sup>−/−</sup>* intestines, suggesting that the molecular alterations in mutant tissues did not simply reflect a generalized cell stress.

To determine whether the NF-κB pathway was required for the increased cytokine production and crypt cell proliferation, we treated *Rab11a<sup>−/−</sup>* organoids with an NF-κB pathway inhibitor, BAY11-0782, which irreversibly inhibits the phosphorylation of IkBα. BAY11-0782 caused significant reductions in IL-6 production and cell proliferation in *Rab11a<sup>−/−</sup>* organoids (Fig 4H and I). These inhibitory effects were as strong as those elicited with an IL-6 neutralizing antibody (Fig 4I). Although IL-6 recombinant proteins stimulated the proliferation of wild-type organoids, the resulting proliferative rate was still lower than *Rab11a<sup>−/−</sup>* organoids (Fig 4I), suggesting that additional cytokines might have also contributed to *Rab11a<sup>−/−</sup>* hyperplasia.

Loss of Rab11a impacted the homeostatic TLR9 intracellular distribution

As microbial sensors in IECs, TLR signaling constitutes the primary link between enteric microbes and epithelial cell-intrinsic NF-κB signaling. The elevated levels of phosphorylated Erk (Fig 4D), another mediator of TLR9 receptor signaling (Lee et al. 2006), and a number of downstream genes of TLR9 signaling, such as defensins and CXCL1 (Lee et al. 2006) (Fig 2C and E), collectively suggested that TLR9 signaling pathway might be activated in Rab11a-deficient IECs. We then explored the potential contribution of abnormal TLR signaling to the observed phenotypes. Despite a normal TLR9 mRNA level detected by microarray and quantitative RT-PCR (Supplementary Fig S4), immunolocalization of TLR9 with a documented antibody (Lee et al. 2004, 2006; Tabet et al. 2006; Palladino et al. 2007) revealed aberrant subcellular receptor aggregations that appeared to be basally (bl) shifted in Rab11a-deficient IECs (bottom panels in Fig 5A and B, and Supplementary Fig S5). Wild-type IECs contained primarily small apical (ap) TLR9 puncta in addition to weak cell surface signals (Lee et al. 2006) (top panels in Fig 5A and B, and Supplementary Fig S5). Close association of TLR9 aggregates with Lamp2<sup>+</sup> (Fig 5B), or with Rab7<sup>+</sup> vesicles (Supplementary Fig S5), was significantly increased in Rab11a-deficient enterocytes, suggesting that an increased amount of TLR9 was retained by endolysosomes.

Western blots for TLR9 detected altered receptor fragmentation patterns in adult *Rab11a<sup>−/−</sup>* intestines (male and female, right panel in Fig 5C), suggesting a changed receptor proteolytic processing. The detected multiple TLR9 fragments in wild-type intestines might reflect stepwise receptor processing by cathepsin and asparagines endopeptidase (Ewald et al. 2011). Using a stable TLR9 knockout (KD) human colonic epithelial Caco2 cell line, we validated the specificity of these endogenous TLR9 fragments (Supplementary Fig S6). This different TLR9 fragmentation pattern was absent in fetal *Rab11a<sup>−/−</sup>* intestines (E15.5, left panel in Fig 5C), but emerged from neonatal stages (P6: left panel in Fig 5C; 2-weeks: Fig 5D and Supplementary Fig S6), when luminal microbiota colonized the intestine. We then performed intracellular vesicle fractionation by sucrose density gradient centrifugation to determine the steady-state distribution of TLR9. In wild-type intestines, TLR9 predominantly coexisted in subcellular fractions containing Rab11a vesicles (left panel, Fig 5D); in Rab11a’s absence, TLR9 distribution shifted toward fractions containing late endosomes (Rab7) and lysosome (Rab9) (right panel, Fig 5D). Using co-immunoprecipitation (co-IP), we detected increased TLR9 retention by Rab7 endosomes and an elevated TLR9-MyD88 interaction in *Rab11a<sup>−/−</sup>* mouse intestines (see arrows, Fig 5E), suggesting that in the absence of Rab11a vesicles, increased amount of TLR9 was contained by late endosome and/or lysosome, where TLR9 was reported to be processed and activated in innate immune cells (Ewald et al. 2008; Park et al., 2014).
Figure 3. Inducible Rab11 depletion in Drosophila enterocytes caused stem cell hyperplasia and cytokine activation.

A Cell type-specific Rab11 knockdown in Drosophila midguts was achieved using promoter-specific Gal4 drivers shown in red. IESC: intestinal epithelial stem cell, EB: enteroblast, EE: enteroendocrine cell, EC: enterocyte.

B Entire midguts of control RNAi and Rab11 RNAi flies were counted for pH3+ mitotic cells. Myo1A-Gal4-driven enterocyte-specific Rab11 knockdown induced the greatest IESC division. ***P < 0.001.

C Similar phenotypes were detected in three independent Rab11 RNAi lines: v108382, v122198, and Trip, all driven by the Myo1AGal4. Representative pHH3 staining is shown for v108382 Rab11 RNAi fly midgut. Arrows point to mitotic cells (red). Nuclei were stained by DAPI in blue.

D–I Confocal images of midguts from control and Rab11 RNAi flies. D, F, H are surface views; and E, G, I are sagittal views. Green cells were Myo1A-Gal4-driven GFP that marked enterocytes. Membrane was stained by β-catenin (Armadillo) antibody (red), nuclear red was Prospero for enteroendocrine cells, and nuclei were stained by DAPI in blue. The arrows point to IESC-EB cell nests encircled by dotted lines. Scale bars, 20 μm.

J Increased Upd3 mRNA level was detected by qRT-PCR in Myo1AGal4-driven Rab11-depleted Drosophila midguts.

K Confocal images of midguts from Upd3-LacZ reporter showed undetectable Upd3 promoter activity in control, but higher reporter activities (red) in Rab11-depleted enterocytes, which were marked by Myo1AGal4-driven GFP (green), suggesting that Rab11 depletion caused cell-autonomous Upd3 activation in enterocytes. Scale bars, 20 μm.
2008). Additionally, when we examined adult germ-free Rab11aIEC mouse intestines where microbial TLR agonists were absent, we observed an identical pattern to the one seen in fetal intestines (Fig 5C and F). These data suggested that Rab11a critically contributed to TLR9 sorting in IECs of conventionally housed, that is, the specific pathogen free (SPF), mice.

Notably, in Caco2 cells that feature mature enterocytes (Peterson & Mooseker, 1992), TLR9 was detected by co-IP to be contained in RAB11A vesicles (Supplementary Fig S7A). Transient knockdown of RAB11A or treatment of cells with monensin, a recycling endosome inhibitor, caused a small but clear alteration of TLR9 fragmentation (Supplementary Fig S7B). Cell surface localization of TLR9 has been reported in both human colon epithelial cells (Lee et al, 2006) and mouse splenic dendritic cells (Onji et al, 2012). Biotinylation and membrane protein isolation assays using stable RAB11A-KD cells detected a 30% reduction of TLR9 being returned to cell surface of RAB11A-KD Caco2 cells (see arrow, Supplementary Fig S7C). In RAB11A's depletion, a sevenfold increase of TLR9 retention by RAB7+ vesicles was observed in these cells (Supplementary Fig S7D), which upon forming polarized cysts, contained a larger acidic endolysosomal compartment (Fig 5G). Quantitative RT-PCR detected a 29-fold increase in IL6 mRNA level in RAB11A-KD Caco2 cells compared to scramble KD cells (Fig 5H). These results suggested that Rab11a-mediated homeostatic TLR9 distribution might be conserved in human colonic epithelial cells.

Rab11a controls epithelial tolerance to TLR agonists

Abnormal TLR9 activation provides potential mechanism for NF-κB activation and inflammatory response observed in Rab11aIEC mice, as compartmentalization of TLR9 in IECs was reported to balance the mucosal tolerance and immune response to microbial stimulations (Wells et al, 2011). It was possible that Rab11aIEC intestinal epithelia were leaky causing bacterial activation of basolateral TLRs such as TLR5 (Rhee et al, 2005), but barrier function analyses using radioactive 3H-L-glucose and 14C-inulin failed to detect leakiness in

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**Figure 4. IEC-intrinsic activation of NF-κB and MAP kinase pathways in Rab11aIEC mice.**

A P65 and Re1B levels were elevated in postnatal Rab11aIEC intestines.
B Inducible Rab11a ablation in Rab11afl/fl;Vil-CreER mice also elevated P65 and Re1B levels.
C P65 and Re1B levels were not elevated in Rab8a knockout intestines.
D Nuclear fractionation assays determined that there was a higher level of nuclear P65 in Rab11aIEC epithelia (15.7%) than control epithelia (9.1%).
E During the development of wild-type mouse intestines, NF-κB and Rab11a elevation showed reverse correlations.
F P65 level was drastically decreased in postnatal villus epithelia, but was continuously activated in postnatal Rab11aIEC epithelia. Scale bars, 20 μm.
G Levels of phosphorylated Erk were increased in Rab11aIEC mouse intestine.
H NF-κB inhibitor BAY11-7082 suppressed IL-6 production from Rab11aIEC organoids. *P < 0.05.
I BAY11-7082 and IL-6 neutralizing antibody suppressed the proliferation of Rab11aIEC organoids. Scale bars, 15 μm. *P < 0.05, ***P < 0.001.

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Figure 5. Rab11a deficiency impacted TLR9 distribution, fragmentation, and activation.

A Immunofluorescent staining for TLR9 (green) detected intraepithelial cell aggregations that shifted basally (bl) in Rab11a-deficient IECs. In wild-type IECs, TLR9 was detected as apical (ap) puncta, in addition to cell surface signals. Lu: lumen. Scale bars, 10 µm.

B Costaining for TLR9 (green) and Lamp2 (red) showed increased TLR9 aggregations in Lamp2+ compartment. Arrows indicate closely associated two signals. Scale bars, 10 µm. ***P < 0.001.

C Western blots for TLR9 showed changed receptor fragmentation pattern in both male and female adult Rab11a−/− mice at SPF conditions. Results represent data from six mice. Note that at E15.5 fetal stage, TLR9 fragmentation pattern was similar between control and mutant intestines. Arrowhead points to an approximately 95 kDa processed TLR9. Lower molecular weight bands (< 40 kDa) may reflect non-specific proteins.

D Subcellular fractionations showed shifted TLR9 compartmentalization in Rab11a−/− IECs toward low sucrose density fractions containing endolysosome. Lane 1–12: 0–50% sucrose fractions (see Materials and Methods). TL: total lysates. XY scatter plots illustrating percentage distribution in each sucrose fraction were generated in Excel on the basis of densitometry measurements by NIH Image.

E Co-IP analyses using mouse intestinal lysates showed increased TLR9 proteins in Rab7a+ vesicles and elevated TLR9-MyD88 association (see arrowheads) in Rab11a−/− compared to control mice. Asterisk indicates small TLR9 fragments present in Rab7a complexes.

F TLR9 fragmentation pattern was similar between control and mutant adult mice at germ-free condition. Note that the patterns in germ-free intestines were similar to those in E15.5 intestines in (C).

G LysoTracker showed that RAB11A-KD Caco2 cysts contained larger acidic endolysosomal compartments. Rainbow color scale identifies acidic compartments as yellow. Scale bars, 15 µm.

H RAB11A-KD Caco2 cells showed increased IL6 and cIAP2 mRNA levels. ***P < 0.001.
Rab11a<sup>ATC</sup> epithelia, which showed rather stronger barrier function than wild-type littermates (Fig 6A), consistent with formation of apical IEC junctions in the mutants (arrows in Supplementary Fig S3B). However, germ-free Rab11a<sup>ATC</sup> mice did demonstrate an overall reduction of crypt cell proliferation (Fig 6B and C) and serum IL-6 levels (Fig 6D) compared with Rab11a<sup>ATC</sup> mice at SPF conditions. Remarkably, germ-free mutants showed reduced mortality and some became capable of breeding (data not shown). Organoids derived from germ-free Rab11a<sup>ATC</sup> mice also showed reduced IL6 expression (Fig 6E), secreted less IL-6 proteins (Fig 6F), and proliferated less (Fig 6G). These data supported the notion that the microbial status influenced the progression of inflammatory phenotype in Rab11a<sup>ATC</sup> mouse intestines.

To determine the specific responses of germ-free Rab11a<sup>ATC</sup> intestinal epithelia to distinct TLR agonists, we performed luminal perfusion analyses in live adult mice using specific ligands for TLR9 (endotoxin-free Escherichia coli DNA) and TLR4 (LPS) (Fig 6H). Both wild-type and Rab11a<sup>ATC</sup> germ-free intestines responded to 4 h of E. coli. DNA perfusion at molecular level, showing increased levels of phosphorylated ERK, IkBα, and p38MAPK, compared with non-perfused counterparts (Fig 6I). Rab11a<sup>ATC</sup> germ-free intestines showed higher levels of ERK phosphorylation, confirming an acute and stronger activation of MAPK pathway in IECs in the absence of Rab11a. Luminal perfusion of TLR9 agonists failed to activate cytokine genes (IL6 and CXCL1) in wild-type germ-free mice (Fig 6J), but drastically activated IL6 and CXCL1 levels (Fig 6J) in Rab11a<sup>ATC</sup> germ-free mice, suggesting that in the absence of Rab11a, IECs could not tolerate apical TLR9 ligand loading. Both wild-type and Rab11a<sup>ATC</sup> germ-free intestines responded to LPS perfusion, whereas the latter showed much more pronounced cytokine responses (Fig 6J), consistent with previous reports that the immune-suppressive function of apical TLR9 plays a role in dampening other TLR agonist stimulations (Lee et al, 2006). Based on these data, we proposed that Rab11a deficiency impaired IEC’s tolerance to microbial TLR agonists.

**Discussion**

Our studies provided genetic evidence for the contribution of Rab11 endosom to intestinal host-microbial homeostasis. Using cell type-specific inducible gene ablation, we demonstrated that Rab11a-deficient mouse and Drosophila enterocytes activated inflammatory signaling pathways and overproduced cytokines, causing IBD phenotypes in both species. Luminal perfusion of germ-free live mouse intestines with distinct microbial TLR agonists demonstrated that Rab11a deficiency broke the mucosal tolerance to TLR agonists. These data suggested an evolutionarily conserved function of Rab11a endosomal compartment in control of intestinal host-microbial interaction.

The endosomal gene network associated with Rab11a-mediated trafficking activity is upregulated during terminal differentiation of the mouse intestinal epithelial cells (Gao & Kaestner, 2010). Expression and functional activation of this important membrane trafficking process may reflect the increased demand by the postnatal IECs that encounter and adapt to the large microbial population after birth. At steady-state conditions in wild-type mice, the direct and constitutive recruitment and sorting of microbial sensors by Rab11a endosome may serve another important immune regulatory function dampening microbial receptor induced immune response to normal microbiota (Fig 7A). Rab11a was reported to influence cell junction in vitro (Wang et al, 2000; Desclozeaux et al, 2008), therefore, we initially suspected that the epithelial leakiness in these mutant mouse intestines might be an enteritis-triggering factor (Su et al, 2009); however, this hypothesis was ruled out by barrier function tests. Somewhat surprisingly, Rab11a<sup>ATC</sup> intestines exhibited even stronger barrier function than wild-type littermates. In fact, loss of Rab25, a member of the Rab11 subfamily also increased Claudin-1 expression and trans-epithelial resistance (Krishnan et al, 2013), consistent with our data suggesting that the epithelial barrier function in Rab11a<sup>ATC</sup> intestine is not impaired.

In cultured human IECs, apical versus basolateral compartmentalization of TLR9 has been linked to the distinct immune responses elicited by ligand-activated TLR9 receptors (Lee et al, 2006). Indeed, in our perfusion assays, wild-type germ-free mouse intestines were largely tolerant to luminal loading of purified TLR9 agonists, as no transcriptional activation of IL6 and CXCL1 was detected. This tolerance appeared to be TLR9-specific, since luminal LPS loading activated both genes in wild-type intestines. However, in the absence of Rab11a, luminal loading of TLR9 agonists dramatically induced IL6 and CXCL1 expression, suggesting that in wild-type IECs, the Rab11a vesicles suppressed TLR9 activation. Since TLR9 activation from the apical surface induced IEC tolerance to subsequent TLR agonist stimulation (Lee et al, 2006), complete loss of this protective mechanism in Rab11a-deficient IECs may be reflected by the strong cytokine response induced by LPS. Alternatively, Rab11a has been reported to recruit TLR4 to phagosomes activating type I interferon response in human monocytes (Husebye et al, 2010). We, at this moment, could not exclude the possibility of pro-inflammatory TLR4 signaling due to missorting of TLR4 in Rab11a knockout tissues. Abnormal TLR4 signaling was reported in cystic fibrosis model (Bruscia et al, 2011). As luminal perfusion of bacterial DNA did induce weak activation of Erk in wild-type intestines, we speculate that Rab11a sorting vesicles might be essential to control the strength of receptor signaling.

Endolysosomal control of TLR9 proteolysis and activation has been described in innate immune cells, but has not been well explored in IECs. Adult Rab11a-deficient IECs showed clearly altered fragmentation patterns compared to controls. In fact, independent intestines from various mutant animals, regardless of their genders, demonstrated strikingly similar fragmentation pattern to each other. This implied that, in Rab11a’s absence, TLR9 was consistently transported into a certain compartment where it was improperly processed. In macrophages, the ectodomain of TLR9 is cleaved in endolysosomes for activation (Ewald et al, 2008; Park et al, 2008). Although the full-length receptor is also capable of ligand binding, only the processed TLR9 recruits MyD88 on activation (Ewald et al, 2008; Park et al, 2008). A recent study further suggested that an association between the cleaved N-terminal part and the truncated TLR9 was critical for ligand sensing and receptor activation (Onji et al, 2012). Rab11a deficiency impacted both TLR9 distribution and fragmentation, accompanied by increased TLR9-MyD88 association and activated TLR9 downstream targets, strongly suggesting that TLR9 was mis-activated in mutant IECs. Indeed, TLR9 recruitment into autophagosome was reported to activate MAP kinases and hypersensitivity in B cells (Chaturvedi et al, 2008). TLR9 transmembrane
**Figure 6. Rab11a controls epithelial tolerance to microbial TLR agonists.**

A Barrier function tests showed that Rab11a<sup>SEC</sup> epithelia were not leaky. The active accumulation of permeabilized radioactive molecules was shown as a ratio of inside/outside quantity of <sup>3</sup>H-L-glucose or <sup>14</sup>C-inulin. Rab11a<sup>SEC</sup> duodenum and ileum showed stronger barrier function than wild-type littermates. ***P < 0.001.

B, C Germ-free Rab11a<sup>SEC</sup> mice showed reduced crypt hyperplasia compared to SPF Rab11a<sup>SEC</sup> mice. Scale bars, 10 μm. **P < 0.01, ***P < 0.001.

D, E Germ-free Rab11a<sup>SEC</sup> mice had decreased levels of serum IL-6 and intestinal IL6 mRNA compared with SPF Rab11a<sup>SEC</sup> mice. *P < 0.05, **P < 0.01.

F, G Rab11a<sup>SEC</sup> organoids derived from germ-free mice showed decreased IL-6 production and reduced proliferation, compared with SPF Rab11a<sup>SEC</sup> mice. *P < 0.05, **P < 0.01, ***P < 0.001.

H Luminal perfusion assays, using specific TLR agonists (E. coli DNA or LPS), were performed on 4-week-old germ-free live animals: Rab11a<sup>SEC</sup> and wild-type littermates. After perfusion, the perfused intestinal tissues (red) and the immediately proximal non-perfused tissues (dotted black lines) were dissected for mRNA and protein analyses.

I Western blots for various signaling pathway effectors. TLR9 ligand-perfused Rab11a<sup>SEC</sup> intestines showed strong phosphorylation of ERK, compared with wild-type or non-perfused tissues.

J qRT-PCR for IL6 and CXCL1 was performed on TLR9 ligand-perfused wild-type and Rab11a<sup>SEC</sup> intestines. IL6 and CXCL1 expression levels in corresponding non-perfused tissues were used as baselines for each genotype. Data are shown as fold inductions. Note that TLR9 ligand perfusion decreased IL6 and CXCL1 expressions in wild-type intestines, whereas the same perfusion induced 128-fold and 64-fold increases of IL6 and CXCL1 levels in Rab11a<sup>SEC</sup> intestines, respectively.
mutations that bypass receptor proteolysis could be activated by self-DNA in dendritic cells causing lethal inflammation in mice (Mouchess et al., 2011). These scenarios, somewhat similar to what was observed in Rab11a-deficient IECs, suggested that intrinsic defects altering TLR9 sorting and processing could induce strong inflammatory responses. In Rab11a-deficient IECs, full activation of TLR9, still required its agonists, since complete removal of microbial agonists (germ-free condition) alleviated inflammatory responses in these mutant mice. However, TLR9 might be the major, but unlikely the sole, microbial receptor trafficked by Rab11a vesicles in enterocytes; therefore, future work is necessary to determine the exact sorting and processing defects in TLR9 and potentially other microbial receptors. Our data at least supported the notion that, at steady-state conditions, Rab11a vesicles favored proper traffic and activation of TLR9 in host enterocytes in constant contact with microbiota (Fig 7A).

Deletion of Rab11a in both mouse and Drosophila enterocytes induced non-autonomous cell division in the IESC compartment. This enterocyte-to-stem cell signaling appears to be mediated by enterocyte-originated cytokines. The similarities shared by both animal species hinted a well-conserved Rab11a-dependent innate mechanism employed by enterocytes to support the host-microbial homeostasis.

**Materials and Methods**

**Mice**

The mouse Rab11a conditional floxed allele was derived through homologous recombination in mouse embryonic stem cells at a 129 genetic background. A loxP site was inserted at 242 bp downstream of Rab11a’s second exon, while a second loxP site introduced at 210 bp downstream of fourth exon. Lgr5^GFP-IRESCreERT2, Vil-Cre, Vil-CreER, Rab8a knockout mice have been described (Madison et al., 2002; el Marjou et al., 2004; Barker et al., 2007). Germ-free colonies were re-derived from plug mating females, housed, and bred in filtered air, autoclaved caging, bedding, food and water. Germ-free status was confirmed by PCR analyses for fecal 16rDNA. Data for mouse experiments were obtained from five to eight individual mice for each genotype group. All experiments were
performed on littersmates unless otherwise stated. Additional molecular and phenotypic analyses are in the Supplementary Information and were detailed previously (Gao et al., 2009; Gao & Kaestner, 2010; Sakamori et al., 2012).

**Drosophila**

All *Drosophila* stocks were maintained at room temperature in yeast extract/cornmeal/molasses/agar food medium. w^{1118} were used as the wild-type for crossing with various Gal4 lines for control experiments. The fly stocks Rab11^{TRIP} was originated from Transgenic RNAi Project of Harvard Medical School and obtained from Bloomington (#27730). The Rab11^{1221/98} and Rab11^{105892} were obtained from Vienna Drosophila RNAi Center. The Gal4 drivers have been described previously (Micchelli & Perrimon, 2006; Zeng et al., 2010; Jiang et al., 2011). The Upd3-LacZ was as described (Zhou et al., 2013). Control flies were crosses between w^r and the Gal4 lines as indicated. All fly crosses also contained the tubulin-Gal80° for temporal control, and 5- to 7-day-old flies were shifted to 29°C for 4 days to allow Gal4 activity and RNAi.

**Mouse intestinal crypt isolation and organoid culture**

Procedures for crypt isolation and organoid culture were essentially the same as reported (Sato et al., 2009). Crypts were counted, resuspended in Matrigel (BD Biosciences, #354234), and seeded into 8-well chamber slides, 48-well or 24-well culture plates, depending on the purpose of the study. After Matrigel polymerization, crypt culture medium (Advanced DMEM/F12 containing 50 ng/ml recombinant murine EGF, 1 μg/ml recombinant murine R-Spondin 1, 100 ng/ml recombinant murine Noggin, 1× N2 supplement, 1× B27 supplement, 1 mM N-acetylcysteine, 2 mM GlutaMax, 10 mM HEPES, and 100 U/ml penicillin/100 mg/ml streptomycin) was applied. For *in vitro* treatment of organoids, 200 crypts/wells were seeded in replicates for each genotype. After formation of control and mutant organoids, fresh medium was replaced containing recombinant mouse IL-6 (100 ng/ml), IL-6 neutralizing antibody (1 μg/ml), BAY11-0782 (10 μM), or no supplement. After 24 h, the cultures were used either for cytokine ELISA or for immunofluorescent analyses detailed in Supplementary Information. All assays were independently repeated at least three times.

**Histopathology scoring of intestinal inflammation**

To assess the inflammation of small intestine, a histological scoring system as described previously (Adolph et al., 2013) was conducted on operator blinded histology sections. Briefly, this histological scoring system comprised five histological features which were evaluated semi-quantitatively (0, absent; 1, mild; 2, moderate; 3, severe): mononuclear cell infiltrate (0–3), crypt hyperplasia (0–3), epithelial injury/erosion (0–3), polymorphonuclear cell infiltrates (0–3), and transmural inflammation (0, absent; 1, submucosal; 2, one focus extending into muscularis and serosa; 3, up to five foci extending into muscularis and serosa; 4, diffuse). In addition, one extended factor was derived based on the fraction of bowel length involved by inflammation: (i) < 10%; (ii) 10–25%; (iii) 25–50%; and (iv) > 50%. The histological score was calculated as a sum of five independent parameters multiplied by the extended factor.

**Sucrose density gradient centrifugation**

Procedures were modified from Yao et al. (2009). Small intestinal tissues were suspended in cold detergent-free lysis buffer (100 mM sodium carbonate pH 11, 0.5 mM EDTA, 1 mM phenyl-methanesulfonyl fluoride and 2× protease inhibitor (Roche Diagnostics) and lysed with 50 strokes using tight pestle in glass homogenizer. Homogenates were centrifuged at 3,000 g for 10 min at 4°C to remove nuclei, large cell debris and unbroken cells. The supernatants were adjusted to 50% sucrose by mixing with 90% (w/v) sucrose solution (in 25 mM 4-Morpholineethanesulfonic acid, 150 mM NaCl, 250 mM NaCO3), and loaded to the bottom of 12.5-ml ultracentrifuge tube. Eleven discontinuous sucrose gradients (40, 35, 25, 22.5, 20, 17.5, 15, 12.5, 10 and 5%, and homogenizing buffer) were sequentially layered on top of the lysates. After centrifugation at 100,000 g for 16 h in SW40Ti swinging-bucket rotor (Beckman) at 4°C, 12 fractions, 1 ml of each layer, were collected and stored at −80°C. Twenty microlitre of each fraction as well as total tissue lysates were denatured and subjected to Western blots.

**Intraluminal perfusion assay**

To examine the response of small intestine to specific TLR9 agonist *in vivo*, mice were anesthetized intraperitoneally with 60 mg/kg ketamine and 5 mg/kg xylazine initially, and subjected to midline laparotony for exposure of the entire small intestine with intact blood vessels and nerve connections. Two small incisions were made at the proximal end of jejunum and the distal end of ileum, respectively. Catheters were inserted, secured with surgical thread, and connected to an inflow polyethylene tube. After the contents were flushed, the small intestine was continuously perfused from jejunum to ileum with KRB buffer containing 8 μg/ml of *E. coli* ssDNA (Invivogen, Catalog #trl-ssec) or 2 μg/ml of LPS (Sigma, Catalog #L4391) at a rate of 15 ml/h using a peristaltic pump for 4 h and then harvested for further analysis. The temperature of mouse body and perfusion solution was maintained at 37°C by heating pads/heat lamps and water bath, respectively.

**Statistical analysis**

Data are presented as mean values of 3–6 independently replicated experiments, with error bars representing standard error of the mean (SEM). A two-tailed Student’s *t*-test was used to determine significance of differences. Co-localization of dual fluorescent signals was deduced from confocal microscopic images using Pearson correlation analyses (Bolte & Cordelieres, 2006). Significance was indicated as **" when *P*-value < 0.05, "**" when *P*-value < 0.01, and "****" *P* < 0.001.

**Supplementary information** for this article is available online: http://emboj.embopress.org

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Author contributions

SY, EMB, JRG, TI, and NG conceived the project, designed experiments, and analyzed data; SY, YN, BK, RS, ES, CP, SD, RFP, and VD performed experiments and analyzed data; SY, TI, and NG wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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