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Rab11-mediated trafficking in host-pathogen interactions

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Preface

Many bacterial and viral pathogens block or subvert host cellular processes to promote successful infection. One host protein targeted by invading pathogens is the small GTPase Rab11 that functions in vesicular trafficking. Rab11 acts in conjunction with a protein complex known as the exocyst to mediate terminal steps in cargo transport via the recycling endosome to cell-cell junctions, phagosomes, and cellular protrusions. These processes contribute to host innate immunity by promoting epithelial and endothelial barrier integrity, sensing and immobilizing pathogens, and repairing pathogen-induced cellular damage. In this Review, we discuss the various mechanisms that pathogens have evolved to disrupt or subvert Rab11-dependent pathways as part of their infection strategy.

To establish infection, pathogenic microorganisms have evolved myriad strategies to circumvent host defenses and to exploit the host cellular machinery. Specific virulence factors disable or subvert vesicular trafficking pathways to and from the host cell surface, which promotes pathogen entry, replication or escape.

Key regulators of various membrane trafficking events are Rab GTPases (reviewed in ¹). They belong to the large protein family of small GTPases and comprise 30–70 members in different organisms. Other small GTPase subfamilies include Ras, Rho/Rac, Arf and Ran, as well as the more recently characterized Rheb, Rad and Rit subfamilies. Binding of GTP to small GTPases stimulates their effector functions, whereas association with GDP inactivates them (**Fig. 1a**). Guanine nucleotide exchange factors (GEFs) stimulate GDP release and act as positive regulators, while GTPase activating proteins (GAPs) inhibit GTPase activity by promoting hydrolysis of GTP to GDP. In addition, GDP dissociation inhibitory proteins (GDIs) bind GDP-bound Rabs to sequester them in the cytoplasm, and opposing GDI displacement factors (GDFs) re-deliver GDP-Rabs to the membrane (**Fig. 1a**).

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Rab11 mediates several cellular processes that involve intracellular vesicle trafficking, including the delivery of plasma membrane proteins to specialized sites (for example, bud sites in yeast or cell–cell junctions in mammalian cells), secretion of various factors (such as growth factors and other peptides, including interferons, cytokines, BMP and transferrin receptor, establishment of cell polarity and targeting of proteins to organelles and cell compartments, which leads to the formation of edges of migrating cells and the midbody during cytokinesis (reviewed in ^{2, 3}). Of note, Rab-11-mediated targeting of proteins to organelles such as cilia ^{4, 5} or tubular structures ⁶ also requires the activation of the downstream Rab GTPase Rab8, which may reflect the ancestral state of the pathway since this organization is also found in yeast ⁷. Furthermore, Rab11-mediated trafficking events also have a role in several aspects of innate immunity, including promoting epithelial and endothelial barrier integrity, targeting of proteins such as TLR4 to phagosomes for receptor-mediated phagocytosis of pathogens⁸, and expulsion of pore-forming toxins from the cell surface ⁹. Also the exocyst, an eight-protein complex (see below) that mediates many Rab11 functions, has a key role in initiating interferon (IFN) signalling in response to double-stranded DNA ¹⁰, regulating autophagy versus INF signalling ¹¹ (**Box 1**).

One prominent trafficking pathway that pathogens modulate or exploit by various mechanisms is the final step of endocytic recycling, at which cargo containing vesicles dock at the cell surface.

Endocytic recycling of internalized extracellular molecules or membrane proteins is initiated by Rab5, which directs plasma membrane-derived vesicles to Rab4+ early endosomes (**Fig. 1b**). Some cargo proteins can then recycle back to the plasma membrane from the early endosome via two pathways: a fast, direct route that depends on Rab4; or a slow route via Rab11+ late recycling endosomes. Rab11+ late recycling endosomes are delivered to the apical cell surface along radial actin filaments by the motor protein myosin V (reviewed in ²), and then tethered at the plasma membrane by binding of Rab11 and myosin V to the exocyst component Sec15, forming a tripartite docking complex. The exocyst is an octameric protein complex (comprising Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, Exo74) that interacts with SNARE complexes and subsequently mediates vesicle fusion and delivery of cargo proteins to the plasma membrane.

Acting in opposition to Rab11-mediated delivery of proteins to cell junctions, the small GTPase Arf6 promotes junctional disassembly in response to various acute inflammatory or vascular permeabilizing factors signals. In addition, Arf6 binds to the exocyst components Sec10 ¹² and Exo70 ¹³ although the function of these direct links between Arf6 the exocyst remain to be explored.

As epithelial and endothelial barriers provide a first line of defense against invasion by microbial pathogens, it is perhaps not surprising that several prominent pathogens secrete virulence factors that block Rab11-mediated trafficking to cell junctions. These activities compromise the integrity of membrane barriers and help pathogens gain access to the host and/or causes tissue breakdown (for example, vascular leakage¹⁴ and general tissue disintegration in anthrax and intestinal fluid loss in cholera infections¹⁵).

Certain pathogens also exploit or subvert Rab11 trafficking; for example, *Salmonella typhimurium* uses the exocyst to recruit new membrane material to sites of bacterial attachment (known as the invadosome to enhance invasion efficiency¹⁶, whereas some viruses, such as Hantavirus, hijack recycling endosomes to bud or be released from cells^{17, 18}. Other pathogens, including *Shigella flexneri*, disrupt trafficking of cadherins and potentially of secreted factors such as antimicrobial peptides from the Golgi to recycling endosomes¹⁹, whereas *Chlamydia* spp. exploit Golgi-derived membranes to create a specialized compartment suitable for their development²⁰.

In this Review, we first discuss the insights that have been gained into the pathogenic roles of anthrax and cholera toxins in the inhibition of Rab11-dependent junctional trafficking. Next we consider how several additional pathogens exploit Rab11-mediated trafficking to promote bacterial invasion and development. Finally, we examine subversion of exocyst-mediated trafficking as a viral exit strategy.

Inhibition of junctional transport

Bacillus anthracis and *Vibrio cholerae* hold important places in the history of infectious disease. Robert Koch demonstrated that *B. anthracis* is the causative agent of anthrax in 1876²¹, and he identified cholera toxin as a ‘poisonous factor’ produced by *V. cholerae* in 1884²². Louis Pasteur elucidated many aspects of anthrax pathogenesis and transmission²³, ultimately developing a crude but effective vaccine²⁴ that ushered in the modern era of medical microbiology. Both of these pathogens secrete exotoxins that lead to overproduction of intracellular cyclic AMP and thereby provoke the pathognomonic features of anthrax and cholera disease.

Anthrax toxins disrupt Rab11-mediated trafficking

Anthrax toxins have a bipartite structure in which a shared B-subunit, protective antigen (PA) mediates entry of the catalytic subunits, edema factor (EF) and lethal factor (LF) into host cells (**Fig. 2a**). EF is a highly active calmodulin-dependent adenylate cyclase and LF is a metalloprotease that cleaves and inactivates host MEKs (reviewed in^{25, 26}) (**Fig. 2a**). EF and LF are essential for pathogenesis of *B. anthracis* as highlighted in classic experiments by Smith and Keppie demonstrating that guinea pigs inoculated with *B. anthracis* were refractory to antibiotics after passing a critical point during infection²⁸. Importantly, they also showed that this lethality could be attributed to accumulation of anthrax toxins, as immunization prior to infection with the anthrax toxin B-subunit was protective, which has also been shown to be effective in humans²⁹.

Anthrax toxins have several important roles during *B. anthracis* infection (reviewed in²⁵) (**Fig. 2a**). During the early, nearly asymptomatic prodromal stages of *B. anthracis* infection, toxins are essential for ‘disarming’ immune cells and promoting transport of spores from the lung to lymph nodes. A recent study reported that mice lacking the key anthrax toxin receptor CMG2 in myeloid cells are resistant to infection by the attenuated non-capsulated Sterne strain of *B. anthracis*²⁷. These mice, however, remain fully sensitive to lethality caused by injection of edema toxin (comprising EF plus protective antigen) or lethal toxin (comprising LF plus protective antigen), which indicates that the toxins exert their lethal

effects on other cell types (see below). During the fulminant stages of *B. anthracis* infection, flu-like symptoms develop, and the disease progresses rapidly, as the microbial toxins facilitate bacterial dissemination throughout the body and disrupt the function of nearly every organ system. EF plays the central role in producing edema, hypotension and the loss of vascular endothelial barrier integrity, which can lead to shock-like death and accounts for a significant fraction of anthrax mortality in humans (reviewed in ²⁵). Anthrax toxins act cooperatively to induce lethality and edema during infection of mice with *B. anthracis* ²⁸. The primary targets of LF and EF in mice responsible for late stage lethality were determined by cell-type specific expression or deletion of the CMG2 receptor to be the cardiovascular system (heart and smooth muscle cells) and the liver, respectively ²⁹. The exact mechanisms that lead to the diverse effects of anthrax toxins that have been observed in different cell types remain elusive and future studies are required to determine whether inhibition of host endocytic recycling contributes to these differences.

Although the structures and biochemical activities of anthrax toxins are well known their cellular effects have been harder to pin down. Expression of the enzymatic subunits of these toxins in the developing *Drosophila melanogaster* wing using the GAL4/UAS system ³⁰, which bypasses the need for receptor-mediated toxin entry yielded the expected effects of anthrax toxins (for example, growth inhibition by LF and cAMP-dependent PKA activation by EF)³¹. In addition, these experiments revealed a novel cooperative activity of the two toxins in blocking Notch signaling ¹⁴: Both toxins reduce cell surface expression of the Notch ligand Delta by inhibiting its endocytic recycling to adherens junctions ¹⁴, a process necessary for Notch receptor activation on adjacent cells ³². In EF-expressing cells, the levels of Rab11 are decreased and endocytic recycling is inhibited, suggesting that EF targets Rab11 to block endocytic recycling. Consistent with this finding, a screen of dominant negative (DN) forms of all 31 *D. melanogaster* Rab proteins showed that only expression of DN-Rab11 mimics the effect of EF. Conversely, in both flies and human cells, over-expression of Rab11 rescues the junction disrupting effects of EF ¹⁴. Although LF does not reduce levels of Rab11, it does reduce apical levels and function of the Rab11 mediator and binding partner Sec15 ¹⁴, an effect that cannot be rescued by Rab11 over-expression. EF also reduces apical Sec15 levels; however, in this case the effect seems to be mediated indirectly by its reduction of Rab11 activity, as expression of DN-Rab11 causes a similar reduction in Sec15. Thus, EF acts by down-regulating Rab11 levels and blocking vesicle formation (and thereby also reducing apical Sec15), whereas LF reduces apical levels of Sec15 in a Rab11 independent fashion. Inhibition of endocytic recycling by either toxin also results in reduced levels of the cell-cell adhesion protein E-cadherin at adherens junctions. Similar effects of both EF and LF have been observed in human brain microvascular endothelial cells in which the toxins block Rab11- and exocyst-mediated trafficking, reduce cadherin levels at junctions, inhibit Notch signaling, and compromise barrier integrity (in transwell assays and *in vivo* in mice), with EF playing a predominant role. Future experiments are required to determine the intersecting mechanisms by which EF acting via increased cAMP (see above) and LF acting via inhibition of MEKs disrupt Rab11- and exocyst-mediated junctional trafficking.

Cholera toxin disrupts intestinal barrier integrity

Cholera toxin (**Fig. 2b**) causes the severe watery diarrhea associated with cholera³³, and its mechanisms of action have been elucidated. Following translocation into the cytoplasm (mediated by the cholera toxin B subunit, CtxB), the enzymatic moiety CtxA (an ADP ribosylase; referred to as Ctx hereafter), binds to the host cofactor ARF6-GTP (which interestingly also acts in opposition to Rab11 to promote junction disassembly (see above)). Ctx transfers ADP-ribose from NAD to the alpha subunit of the stimulating G-protein (Gsq) which causes a pathological increase in cellular cAMP levels through the induction of host adenylate cyclases at the plasma membrane (reviewed in REFS^{33,34}). Increased cAMP levels act via protein kinase A (PKA) to induce the ion channel cystic fibrosis transmembrane receptor (CFTR), which leads to Cl⁻ ion secretion into the intestinal lumen (reviewed in³⁵). This transcellular secretion of Cl⁻ ions is accompanied by paracellular efflux of Na⁺ ions and water to preserve electroneutrality and osmotic balance, resulting in a large volume of fluid loss (up to 10-20 liters per day). This model of Ctx action is supported by a wealth of experimental evidence, a definitive example being the failure of purified cholera toxin to induce fluid secretion in the small intestine of CFTR^{-/-} null mice³⁶. Until recently, however, the mechanisms by which paracellular efflux of Na⁺ ions and water occurs had remained unclear.

As both Ctx and EF induce pathological levels of cAMP, the question arose whether Ctx might also disrupt cell junctions by inhibiting Rab11-mediated trafficking. Such an effect would facilitate the efflux of Na⁺ ions and water via a paracellular route that accompany transcellular Cl⁻ secretion to generate the profuse diarrhea caused by cholera. In agreement with this hypothesis, junctional levels of Rab11, Sec15, and E-cadherin are greatly reduced by Ctx treatment of human intestinal epithelial cell lines¹⁵ (**Fig. 2b**). Ctx also alters tight junctions, disrupting their normal strict alignment with the more basal adherens junctions, and inhibits Notch signaling. Similar effects are also observed *in vivo* in murine ligated ileal loop preparations where Ctx induces fluid secretion, down-regulation of Rab11 and Sec15 levels, and creates apical gaps between cells (**Fig. 2c**). Also, in the fly gut, Ctx causes weight loss, dye leakage, large lacunal gaps between cells that are visible at the ultrastructural level, and lethality, all of which can be rescued by Rab11 over-expression. Conversely, expression of a dominant-negative form of Rab11 mimics Ctx effects causing breakdown of intestinal barrier integrity as judged by the ability of ingested dye to leak into the body cavity¹⁵. Junctional defects caused by Ctx in the fly gut and human cells are remarkably similar to those previously reported in small intestine biopsies from human cholera patients³⁷ (**Fig. 2c**). As with purified toxin, the Ctx-dependent lethality and junctional defects seen upon *V. cholerae* infection of adult flies can be mitigated by over-expressing Rab11 in the midgut (the fly small intestine equivalent). In both flies and human cells, evidence suggests that the effects of Ctx are mediated by a combination of the two primary known cAMP effectors, PKA and EPAC (a GEF for the small GTPase Rap1, which has several known links to the exocyst). Activation of either effector pathway by genetic means in flies or using pathway specific cAMP analogs in mammalian cells also disrupts cell junctions, whereas chemical inhibition of either PKA or EPAC reduces the effects of Ctx¹⁵. These observations indicate that Rab11-dependent endocytic recycling helps

maintain normal intestinal barrier integrity and that boosting this process offers protection against the junctional disruptive effects of Ctx and infection with *V. cholerae*.

How pathological levels of cAMP, via PKA and EPAC lead to the down-regulation and inhibition of Rab11 in the vascular endothelium (EF) or gut (Ctx) is a central question for future research. It is conceivable that the observed decrease in Rab11 protein levels is caused by increased protein turnover (for example, proteasome-dependent degradation of Rab11), alterations in membrane compartment identities (for example, elimination of the late recycling endosome compartment), or reduced production of proteins (for example, inhibition of Rab11 mRNA transcription or mRNA instability) or a combination thereof. Other pathogens that liberate cAMP-producing toxins (reviewed in ³⁸) may possibly inhibit Rab11 activity to disrupt host cell barriers. Prominent examples of such pathogens include toxigenic *Escherichia coli* (via Ltx, which is highly related to CtxA; and STa, which stimulates host guanylate cyclases to indirectly increase cAMP levels and activates CFTR by cGMP-dependent protein kinase II ; reviewed in ^{39,40}), *Bordetella pertussis* (via CyaA, a CAM-dependent adenylate cyclase that is related to EF; and pertussis toxin (Ptx), which inhibits endogenous adenylate cyclases) and *Pseudomonas aeruginosa* (via ExoY, an adenylate cyclase). Finally, there are surprising parallels between the junction disrupting actions of cAMP toxins and microbial pore-forming toxins that merit further scrutiny (Box 2).

Pathogens exploiting Rab11 trafficking

Various intracellular pathogens evade destruction via phagocytosis by subverting Rab-mediated trafficking events and reprogramming membrane compartment identities to create environments that support survival and replication within host cells (reviewed in ^{41, 42}) (**Fig. 3**). In addition, several pathogens exploit or inhibit the endocytic recycling pathway to invade or escape host cells.

Commandeering the exocyst during bacterial invasion

Salmonella typhimurium is an intracellular pathogen that uses a type III secretion system (T3SS) to inject effector proteins into host cells. The tip of this injection complex comprises two proteins that penetrate into the host cytoplasm. These proteins (SipB and SipC) not only participate in transferring virulence factors into the host cell, but also interact with host factors, including the exocyst, to redirect resources to sites of bacterial invasion. SipC interacts with the plasma membrane-associated component Exo70 to recruit the exocyst complex to sites of *Salmonella* attachment. This might lead to the increased delivery of membrane proteins, which promotes membrane expansion and facilitates bacterial invasion ¹⁶ (**Fig. 3**). At the same time, SopE and SopE2, two injected *Salmonella* effectors, function as GEFs to activate the small GTPase RalA, which can bind in a mutually exclusive fashion to either of the exocyst components Sec5 or Exo84 (**Box 2**). Recruitment of the exocyst and activated RalA is essential for bacterial entry, as RNAi of the exocyst components Exo70 or Sec5 or knockdown of RalA lead to reduced bacterial uptake¹⁷. Association of RalA with the exocyst is important in this process, as the effect of RNAi-mediated depletion of Sec5 can be reversed by over-expressing wild-type Sec5, but not with a Sec5 point mutant defective in binding RalA. SipC also binds to actin and remodels the

apical cytoskeleton⁴³, again providing a link to the exocyst, as this complex affects the apical cytoskeletal architecture. Thus, *Salmonella* uses an orchestrated set of effectors that induce localized membrane expansion and cytoskeleton remodeling to promote its engulfment by host cells.

Shigella flexneri also uses tip proteins (IpaB and IpaC) of a T3SS to penetrate into the host cytoplasm. One of these proteins (IpaB) regulates lipid trafficking. IpaB redirects cholesterol to the site of bacterial entry¹⁹ (**Fig. 3**) by altering the architecture and function of the recycling endosome, causing tubulation of the Rab11+ membrane compartment, although Rab11 is not the direct target of IpaB. Anterograde trafficking of E-cadherin via the Golgi and recycling endosome to cell junctions is greatly reduced by *Shigella* infection. This major disruption of the Rab11+ compartment suggests that trafficking of various secreted factors (for example, cytokines and antimicrobial peptides) and cell surface receptors that are required for defence may also be disrupted during *Shigella* infection. IpaB binds cholesterol and thereby depletes this lipid from the Golgi causing its fragmentation, an effect that can be reversed by co-treatment of cells with IpaB protein plus exogenous cholesterol.

The intracellular pathogen *Chlamydia trachomatis*, which forms inclusion bodies in host cells, also leads to Rab11-dependent Golgi fragmentation, which is required for bacterial development and reproduction²⁰. RNAi screening for host factors required for *C. trachomatis* replication identified Rab11 and Rab6 as essential elements, consistent with a prior study documenting association of both GTPases with this bacterium during infection⁴⁴. Rab11 and Rab6, which act to promote anterograde and retrograde trafficking respectively, are also essential for Golgi fragmentation during infection²⁰ as RNAi knock-down of either GTPase restores compact Golgi morphology and inhibits bacterial replication (**Fig. 3**). The importance of inducing Golgi fragmentation for *C. trachomatis* replication is further substantiated by the observation that RNAi of the resident Golgi protein p155, which alone causes fragmentation of this organelle, can overcome the rescuing effect of RNAi-mediated knockdown of Rab11 knockdown cells and restore development of *C. trachomatis*. Interestingly, RNAi of another Golgi protein, golgin-84, which can also induce Golgi fragmentation, is unable to reverse either the restoration of Golgi structure or bacterial reproduction induced by RNAi-mediated knockdown of Rab11, again linking Rab11-mediated Golgi fragmentation and bacterial replication. These genetic epistasis experiments further suggest that Rab11 functions at a step following that of golgin-84 and before p155 in Golgi-to-endosome, defining a pathway to be dissected in further studies.

Subversion of vesicular trafficking by viruses

An important step in the viral life cycle is exit from the host cell. Some viruses assemble complete particles at the cell surface, whereas in other cases, particles are assembled intracellularly and then trafficked as an intact virus to the cell surface. As an example of the latter strategy, Andes virus (ANDV), a New World hantavirus, exploits Rab11-mediated trafficking for delivery to the apical cell surface (**Fig. 4**). This virus forms cytoplasmic inclusions that localize to both *trans*-Golgi and Rab8+ and/or Rab11+ vesicular compartments¹⁸. ANDV preferentially co-localizes with the inactive GDP-bound form of Rab11 (as well as both GDP- and GTP-bound forms of Rab8) in the perinuclear *trans*-Golgi

compartment where viral assembly is thought to take place. By contrast, active Rab11-GTP, which labels recycling endosomes en route to the cell surface, displays only a partial overlap with ANDV. However, Rab8- and Rab11-mediated transport to the cell surface is essential for egress of ANDV from cells, as inhibition of these Rabs resulted in more than a 10-fold decrease in viral secretion under conditions where viral entry was undisturbed.

Rab11 is also essential for trafficking components of respiratory syncytial virus (RSV) ¹⁷, HIV-1 ⁴⁵, and another retrovirus, Mason–Pfizer monkey virus ⁴⁶, to the cell surface where mature viral particles assemble. For example, RSV, a leading cause of lower respiratory illness in children and the elderly, co-opts the Rab11-dependent apical recycling machinery for exit from the host ¹⁷ (**Fig. 4**). RSV release is strongly apically polarized, even when cultured cells are infected basally. Inhibition of apical endocytic recycling by expression of dominant-negative forms of either the MyoV motor protein or the Rab11 family interacting protein (Rab11-FIP1) severely reduces RSV titers, but did not affect propagation of either vaccinia virus or influenza virus. This disruption of viral replication does not alter the ability of RSV to infect cells, but rather restricts its spread to other cells, presumably by preventing Rab11-dependent transport of viral components to the cell surface.

Conclusions and outlook

Rab11 and the exocyst serve crucial host protective roles contributing to several aspects of innate immunity, including establishment and maintenance of endothelial and epithelial barrier integrity, trafficking of IFN signalling complexes to the phagosomes and acting as a scaffold for building alternative protein complexes to promote innate immune signalling versus autophagy. These functions of Rab11- and exocyst-mediated trafficking, however, represent a double-edged sword, as they can be blocked or subverted by various pathogens.

The cAMP-producing toxins, anthrax EF and cholera Ctx (reviewed in ³⁸) are key virulence factors that disrupt Rab11- and exocyst-mediated trafficking of cargo such as adhesion molecules and signalling complexes, such as Notch ligands, to cell junctions. EF disrupts endothelial junctions, which leads to vascular leakage in mice, whereas Ctx disrupts intestinal epithelial barrier integrity thereby potentially contributing to the massive fluid efflux driving the severe life-threatening diarrhea associated with cholera infection. Whether the reported barrier disruptive effects caused by *P. aeruginosa* ExoY toxin ⁴⁷ or enterotoxigenic *E. coli* heat-labile and heat-stable toxins (reviewed in ^{39, 48}) also act by inhibiting the exocyst and whether cAMP production by CyaA and Ptx during *B. pertussis* infection ⁴⁹ disrupt airway epithelial barrier function are important future questions to address. Similarly, the roles of cAMP-mediated inhibition of Rab11 potentially caused by the intracellular pathogen *M. tuberculosis*, which secretes cAMP into the host cytoplasm ⁵⁰, or by *P. gingivalis*, which exploits host TLR2 signalling to induce endogenous cAMP production, ⁵¹ require further investigation.

Although EF and Ctx can on their own recapitulate key pathologies of anthrax and cholera respectively, these cAMP-producing toxins are not the only potentially relevant virulence factors produced during infection. Indeed, in the case of anthrax, EF acts synergistically with the metalloprotease LF to inhibit Rab11/exocyst-mediated trafficking ¹⁴. Similarly, *Y.*

pestis produces the adenylate cyclase YpAC^{38,52} and several factors that inhibit MEK signalling (for example, YopJ⁵³). It remains to be determined whether synergy between these toxins contributes to pathogenesis of plague. cAMP-producing toxins might also act collaboratively with other toxic factors such as the PFTs of *B. anthracis* (anthrolysin O) and *V. cholerae* (VCC) during infection. Finally, in the case of anthrax, as MAPK p38⁵⁴ and TLR4 signalling⁵⁵ are key host defense responses to PFTs⁵⁴, their inhibition by LF and EF, respectively, may represent important neutralizing effects. Examination of these key nodes should yield fresh insights into cooperative interactions between virulence factors.

Another arena for future investigation is whether other known features of disease caused by cAMP-producing toxins may be result from inhibition of Rab11- and exocyst-mediated functions. For example, during the early and nearly asymptomatic phase of infection, anthrax toxins inhibit various aspects of the immune response, allowing the bacteria to proliferate and disseminate into the circulation (reviewed in^{56,57}). As myeloid cells are crucial targets of anthrax toxins during this phase²⁷, it would be interesting to examine whether inhibition of Rab11 function by anthrax toxins alters cytokine secretion during establishment of infection. Conversely, over-expression of Rab11 might mitigate aspects of toxin-mediated lethality. Similarly, as Ctx has been implicated in disrupting innate immune functions during establishment of infection⁵⁸, how inhibition of Rab11- and exocyst-mediated functions contribute to these role(s) of Ctx should be studied. EF effects on the liver are also of interest, as a recent report found that hepatocytes are a key target of EF-mediated lethality in mice²⁹. Experiments could probe how inhibition of Rab11- and exocyst-mediated trafficking by EF disrupts cell-cell junctions in bile duct cholangiocytes, whether it prevents hyperpolarization of hepatocytes to form canaliculi, or whether this toxin impedes the secretion of key hepatic factors. If such effects are observed, a central question will be how they may contribute to the lethal effects of EF and whether selective over-expression of Rab11 in the liver can reverse these effects and restore viability. As liver pathology does not seem to be a notable feature of lethal anthrax cases in humans⁵⁹, one might ask whether more fine scale analysis in mice can point to specific cellular phenotypes to examine more closely in human samples. Similarly, the cell type or cell types that are essential for generating edema in response to EF (for example, lung edema or footpad swelling) must be identified to examine if such swelling can be countered by restoring endocytic recycling.

Another area for future study is to determine whether pathogens exploit or inhibit additional Rab11- and exocyst-mediated events. cAMP-producing toxins or other pathogenic factors may contribute to disease pathogenesis by inhibiting the establishment of apical-basal polarity (e.g., liver), trafficking to cilia (e.g., kidney or regulation of vascular flow), cell migration (e.g., macrophages) or cell division (for example, intestinal crypt). Pathogens manipulate the switch-like regulatory function of the exocyst in promoting the activation of innate immune signalling (e.g., via ubiquitylated RalB-Sec5-TANK1 complexes) or autophagy (e.g., via RalB-Exo84-beclin complexes). Studies probing these questions may yield exciting discoveries and define new molecular targets for pharmacological intervention.

Box 1**Role of Rab11 and the exocyst in innate immunity**

The small GTPase Rab11 and the exocyst complex are involved in several aspects of innate immunity (see the figure). For example, the transmembrane protein STING (stimulator of interferon genes) senses foreign non-CpG double stranded DNA (dsDNA) inadvertently liberated by pathogens such as lysed bacteria or viruses ⁶⁰ (see the figure, part a). In response to exogenous DNA, STING co-localizes with the exocyst component Sec5 and TANK-binding kinase-1 (TBK1) ¹⁰, which leads to the induction of IFN- β gene expression ⁶¹. The exocyst acts as a hub for the switch-like small GTPase RALB, which, depending on its ubiquitylation state, alternatively engages distinct exocyst components to trigger innate immune signalling (binding of Sec5 when ubiquitylated at lysine 47) or autophagy (when deubiquitylated by USP33, which triggers assembly of a RalB-Exo84-beclin1 complex) ¹¹ (see the figure, part b). Similarly, in addition to contributing to the general process of phagocytic engulfment (reviewed in ⁶²), Rab11 is required for trafficking Toll-like receptor 4 (TLR4) from late recycling endosomes to phagosomes (note that a fraction of TLR4 in phagosomes derives from the plasma membrane in a Rab11-independent manner) to initiate interferon signalling ⁸ (see the figure, part c). Upon binding lipopolysaccharide (LPS) on the surface of Gram-negative bacteria, TLR4 co-localizes with Rab11 in recycling endosomes and translocates together with its transducer TRAM to the phagosome engulfing the pathogen, which leads to the induction IFN- β expression. Last, Rab11-dependent trafficking has an important role in clearing pore-forming toxins (PFTs) from the cell surface and repair of the plasma membrane (see the figure, part d). For example, in *C. elegans*, Rab11 is essential against infection by bacteria secreting PFTs such as nemocidal *Bacillus thuringiensis* Cry5 toxin or VCC from *V. cholerae*. Endocytic recycling clears PFTs from the cell surface both by internalization, presumably followed by degradation in the lysosome by a Rab5- and Rab7-dependent process, and by Rab11-mediated expulsion of apical microvilli¹¹.

Box 2**Similarities between pore-forming toxins and cAMP toxins**

Many microbial pathogens can breach epithelial barriers during infection, and various virulence factors, including proteases and actin-modifying enzymes have been identified that aid in this process ⁶⁵. One particularly intriguing example is *Staphylococcus aureus* α -hemolysin (also known as α -toxin), a pore-forming toxin (PFT) that binds to the ADAM10 metalloprotease as its host receptor ⁶⁶ (see the figure). Binding of α -toxin activates the membrane-associated host metalloprotease ADAM10 ⁶⁶, which cleaves E-cadherin to disrupt junctional integrity ⁶⁶⁻⁷⁰. ADAM10 also cleaves Notch receptors in mammals ⁷¹⁻⁷⁴, (or its orthologue Kuzbanian in flies and nematode worms ^{75, 76}), and thus we speculate that ADAM10-mediated Notch cleavage might have a role in the barrier disruptive effects of α -toxin. There are potentially important parallels between PFTs and cyclic AMP-producing toxins. For instance, in the case of cholera, Ctx increases intracellular cAMP levels, which leads to Cl-ion secretion via PKA-mediated

activation of the CFTR ion transporter, whereas the PFT VCC is itself a Cl⁻ ion conducting pore⁷⁷ that is processed and activated by the ADAM10-related protease ADAM17⁷⁸ (see the figure). Also, as Rab11-dependent endocytic trafficking has a key role in eliminating PFTs from the cell surface (by endocytosis and expulsion of microvilli)⁹, and inhibition of this process by cAMP toxins may augment the effect of the PFTs. This strategy of expelling PFT-infested membrane is has also been observed for *S. aureus* α -toxin⁶³ and the larger PFT streptolysin O⁶⁴ group A *Streptococcus* can similarly be shed via 'toxisome' vesicles from several infected cell types.

Determining whether ADAM17, like ADAM10, cleaves cadherins or Notch receptors and exploring potential links between α -toxin, Notch cleavage and endocytic recycling will be interesting avenues for further investigation. It may also be informative to study other potential parallels between α -toxin and cAMP toxin functions. For example, it is likely that both types of toxins reduce the cellular ATP/AMP ratio, as α -toxin results in ATP efflux through the pore, an effect that contributes to its toxicity⁷⁹, and because high levels of cAMP production also deplete ATP reserves. ADAM-10 also serves as a channel to allow efflux of ATP⁷⁹ (probably evoking host cellular responses via reduction in the ATP/AMP ratio), possibly mediated by effectors such as AMPK. Intriguingly, the anthrax toxin lethal factor (LF) also induces the depletion of ATP from macrophages (via a connexin release channel), which leads to the activation of host immune signalling⁸⁰.

Biographies

Annabel Guichard is a research specialist at UCSD. She trained at the Ecole Normale Supérieure de Lyon, and obtained her Ph.D at the Joseph Fourier University of Grenoble, France. In her postdoctoral studies, she has developed *Drosophila* as a model to understand the mechanism of action of bacterial toxins.

Victor Nizet is Professor of Pediatrics and Pharmacy at UCSD leading the Center for Immunity, Infection and Inflammation. He trained at Reed College, Stanford Medical School, Boston Children's Hospital and the University of Washington. His laboratory conducts interdisciplinary research in bacterial pathogenesis, innate immunity and novel infectious disease therapeutics.

Ethan Bier is a professor in Cell and Developmental Biology at UCSD. He did graduate work in immunology with Allan Maxam at Harvard Medical School and postdoctoral training with Lily and Yuh Nung Jan at UCSF. His laboratory uses *Drosophila* to study neural induction and understand mechanisms of human disease.

Glossary

Adherens Junction	A subapically localized cell-cell junction consisting of transmembrane cadherin adhesion molecules which interact with the cytoskeleton via α and β -catenins and link epithelial and endothelial cells and allow them to form contiguous sheets.
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Golgi trafficking	Vesicles derived from Golgi membranes are directed to the cell surface via anterograde trafficking. In addition, vesicles derived from the cell surface or other intracellular membrane compartments can be directed back to the Golgi via retrograde trafficking.
Invadosome	A subcellular structure deriving from the plasma membrane that mediates bacterial invasion of the host cell.
Notch Signaling	A signaling pathway that controls a myriad of cell fate and growth decisions. It is activated at adherens junctions by cell surface tethered ligands (for example, Delta) on one cell stimulating Notch receptors on adjacent cells.
RalB	A small GTPase that can bind in a mutually exclusive fashion to exocyst components Sec5 or Exo84 to act as a molecular switch between immune signaling and autophagy.
Recycling endosome	A late vesicular compartment that is involved in recycling membrane proteins and <i>de novo</i> synthesized cargo from the Golgi to the cell surface.
SNARE proteins	A family of related proteins that mediate the fusion of surface bound vesicles with the plasma membrane (for example, SNAP25, a plasma membrane tethered SNARE (t-SNARE) and a vesicular SNARE (v-SNARE) form a trimeric complex to initiate membrane fusion).
Tight Junction	The most apically localized cell-cell junction consisting of adhesive claudins and occludin that act as a diffusion barrier to ions, water, and other small molecules.
Toxisome	A vesicle containing toxic factors that is expelled from cell surface (for example, shed microvilli).

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Key Points

1. Bacterial and viral pathogens disrupt or exploit Rab11 and exocyst-dependent vesicular trafficking.
2. cAMP toxins inhibit endocytic recycling of proteins to cell junctions thereby disrupting vascular endothelial and epithelial barrier integrity.
3. Bacterial pathogens exploit endocytic recycling to divert host resources to sites of bacterial invasion.
4. Disruption of trafficking from the Golgi to recycling endosome contributes to pathogen replication.
5. Viruses subvert endocytic recycling to exit from host cells.
6. Innate immune signalling, autophagy and toxin shedding are regulated by the exocyst.

Figure 1. Rab small GTPase mediated endocytic recycling

a) Rab regulatory cycles. Rab-GTPases are regulated by two coupled cycles: guanine-nucleotide exchange factors (GEFs) exchange GDP for GTP to generate the active Rab•GTP bound form, whereas GTPase activating proteins (GAPs) generate the inactive Rab•GDP bound form. GEFs and GAPs can be highly selective for specific Rabs. Rabs are also regulated by extraction from vesicle membranes by cytoplasmic GDP dissociation inhibitory (GDIs) proteins that bind non-selectively to Rab•GDP forms and sequester them in the cytoplasm. GDIs have a hydrophobic pocket that binds the prenylated moiety of Rabs (blue line), which is otherwise inserted into the lipid bilayer of a vesicle. Membrane-bound GDI displacement factors (GDFs) extract Rab•GDP from complexes with GDIs and re-deliver them to the membrane compartments.

b) Rab11-mediated junctional transport. Retrieval of membrane proteins into early endosomes occurs through endocytosis, which is mediated through Rab5 and Arf6, respectively. Cargo from the early endosome can be recycled back to the cell surface by a fast (that is, within minutes) Rab4-mediated pathway and a slow (that is, within hours) Rab11-mediated process via late recycling endosomes. Early endosomes can also traffic cargo via Rab5 to the multivesicular body (MVB; also known as the late endosome) and then via Rab7 to the lysosome. Late recycling endosomes can also acquire cargo via Rab11 from the Golgi. Rab11+ late recycling endosomes are delivered to the cell surface along actin filaments via MyoV motors, where these vesicles are tethered to the plasma membrane by Rab11 binding to the exocyst component Sec15 forming a tripartite docking complex. The late recycling endosome are also decorated with vesicle SNARE (vSNARE) and the SNARE component SNAP25, which binds to the exocyst. Following docking, vSNARE forms a trimeric complex with SNAP25 and the membrane tethered tSNARE to initiate fusion of the late recycling endosome with the plasma membrane and delivery of cargo.

Figure 2. The mode of action of anthrax and cholera toxins

a) *Bacillus anthracis* edema toxin (comprising edema factor (EF) and protective antigen (PA)) and lethal toxin (comprising lethal factor (LF) and PA) bind to the host receptors CMG2 and TEM8, which leads to their endocytic uptake and translocation to the cytoplasm. EF, a calmodulin-dependent adenylate cyclase, remains perinuclear, where it produces cyclic AMP to activate PKA as well as EPAC and Rap1. The metalloprotease LF disperses into the cytoplasm to cleave and inactivate MEKs. These pathways then reduce levels of Rab11 (EF) on late recycling endosomes and of apical Sec15 (EF and LF), thereby blocking exocyst-mediated trafficking of junctional proteins (such as, cadherins and Notch ligands) to adherens junctions. The alignment of tight junctions with adherens junctions might also be altered. These effects of anthrax toxins on membrane junctions result in reduced endothelial barrier function and vascular leakage.

b) The *Vibrio cholerae* toxin holoenzyme consists of the catalytic A-subunit (CtxA) and five B-subunits (CtxB). The toxin binds to the ganglioside GM1 on the plasma membrane, where it is endocytosed. CtxA, is translocated into the cytoplasm, where it binds to the Arf6 and activates host adenylate cyclases to increase cAMP levels and induce PKA as well as EPAC and Rap1. This results in the reduction of junctional levels of Rab11 and the exocyst component Sec15, which inhibits trafficking of cargo to adherens junctions and tight junctions. The junction disrupting effects of CtxA may increase paracellular efflux of Na⁺ and water thereby facilitating transcellular secretion of Cl⁻ ions induced by PKA-dependent phosphorylation of CFTR.

c) Cholera toxin produces apical gaps (arrows) and creates lacuna (asterisks) between intestinal epithelial cells and shortens microvilli in patients infected with *V. cholerae* (top: Modified/Reproduced with permission from Fig. 3A,C in ³⁷), in cholera toxin-treated CACO-2 cells (Modified/Reproduced with permission from Fig. 4A,B in ¹⁵), and in the *Drosophila* gut (Modified/Reproduced with permission from Fig. 7O,P in ¹⁵ – see also supplemental Fig. 7K-P in ¹⁵).

Figure 3. Pathogen subversion of Rab11 and exocyst functions

The intracellular bacterial pathogen *Salmonella* uses a type III Secretion System (T3SS) to inject virulence factors into the host cell. The tips of the T3SS comprises two proteins that penetrate into the host cytoplasm, namely SipB and SipC. These proteins which penetrate into the cytoplasm, also interact with host proteins to manipulate membrane trafficking. SipC interacts with the exocyst component Exo70 to recruit membrane to sites of bacterial invasion (invadosomes). The *Salmonella* T3SS also injects the effectors SopE and SopE2. These proteins are RalA GEFs that promote an interaction between RalA and Sec5, which is essential for bacterial invasion. *Shigella* uses the T3SS proteins IpaB and IpaC to penetrate host cells. IpaB binds to and redirects cholesterol, to the site of bacterial entry from the Golgi, leading to Golgi fragmentation. Golgi fragmentation leads to tubulation of the Rab11 compartment and disruption of E-cadherin transport to adherens junctions and thus junctional disruption. *Chlamydia*, another intracellular pathogen, also induces Golgi fragmentation via interactions with Rab11 and Rab6 to disrupt anterograde (Rab11 mediated) and retrograde (Rab6-mediated) transport between the Golgi and late recycling endosomes. This promotes *Chlamydia* development and reproduction. Golgi fragmentation through Rab11 also requires the resident Golgi protein p155.

Figure 4. Viral exit via the recycling endosome

Andes virus (ANDV), a New World hantavirus, forms cytoplasmic inclusions and undergoes assembly in the *trans*-Golgi (Rab11•GDP+) and recycling endosomal (Rab11•GTP+) compartments. Mature viral particles are then trafficked via the recycling pathway in a Rab11-dependent fashion to the cell surface where they are released. Respiratory syncytial virus (RSV) enters host cells either apically or basally and is then exported to the apical cell surface via Rab11 and MyoV-dependent trafficking.

Box1. Role of Rab11 and the exocyst in innate immunity

Box2. Similarities between pore-forming toxins and cAMP toxins