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Authors

Xu, Jin
Toops, Kimberly A
Diaz, Fernando
[et al.](#)

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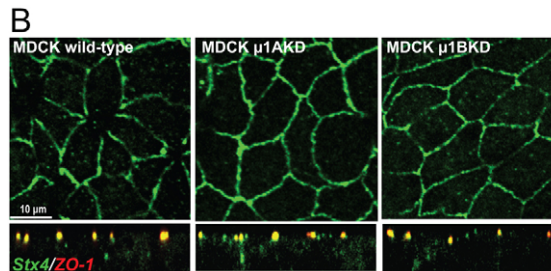
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There was an error published in *J. Cell Sci.* **125**, 5937–5943.

In Fig. 4B the same image was inadvertently presented as the *xy* section (top panels) of syntaxin 4 staining in MDCK wild-type and MDCK μ 1A-KD cells. In the correct version (shown below), the appropriate *xy* image for μ 1A-KD (top panel, middle) matches the corresponding *xz* panel (bottom panel) that was part of the original figure.

The mistake in the figure did not affect the conclusions of the paper.

The correct Fig. 4B is shown below.



The authors apologise for this mistake.

Mechanism of polarized lysosome exocytosis in epithelial cells

Jin Xu¹, Kimberly A. Toops¹, Fernando Diaz², Jose Maria Carvajal-Gonzalez², Diego Gravotta², Francesca Mazzoni², Ryan Schreiner², Enrique Rodriguez-Boulan^{2,3,*} and Aparna Lakkaraju^{1,*}

¹Department of Ophthalmology and Visual Sciences, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI 53705, USA

²Margaret M. Dyson Vision Research Institute, Department of Ophthalmology, Weill Medical College of Cornell University, New York, NY 10021, USA

³Department of Cell and Developmental Biology, Weill Medical College of Cornell University, New York, NY 10021, USA

*Authors for correspondence (boulan@med.cornell.edu; lakkaraju@wisc.edu)

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Summary

Fusion of lysosomes with the plasma membrane is a calcium-dependent process that is crucial for membrane repair, limiting pathogen entry and clearing cellular debris. In non-polarized cells, lysosome exocytosis facilitates rapid resealing of torn membranes. Here, we investigate the mechanism of lysosome exocytosis in polarized epithelia, the main barrier between the organism and the external environment and the first line of defense against pathogens. We find that in polarized Madin-Darby canine kidney (MDCK) cells, calcium ionophores or pore-forming toxins cause lysosomes to fuse predominantly with the basolateral membrane. This polarized exocytosis is regulated by the actin cytoskeleton, membrane cholesterol and the clathrin adaptor AP-1. Depolymerization of actin, but not microtubules, causes apical lysosome fusion, supporting the hypothesis that cortical actin is a barrier to exocytosis. Overloading lysosomes with cholesterol inhibits exocytosis, suggesting that excess cholesterol paralyzes lysosomal traffic. The clathrin adaptor AP-1 is responsible for accurately targeting syntaxin 4 to the basolateral domain. In cells lacking either the ubiquitous AP-1A or the epithelial-specific AP-1B, syntaxin 4 is non-polar. This causes lysosomes to fuse with both the apical and basolateral membranes. Consistent with these findings, RNAi-mediated depletion of syntaxin 4 inhibits basolateral exocytosis in wild-type MDCK, and both apical and basolateral exocytosis in cells lacking AP-1A or AP-1B. Our results provide fundamental insight into the molecular machinery involved in membrane repair in polarized epithelia and suggest that AP-1 is a crucial regulator of this process.

Key words: Lysosome, Polarity, Exocytosis, Membrane repair, AP-1 clathrin adaptor, SNARE protein, Fusion

Introduction

Lysosomes, long thought to be little more than terminal degradative compartments of the cell, are now recognized as central players in maintaining cellular homeostasis (Luzio et al., 2007). Melanocytes, platelets and cytotoxic T lymphocytes (CTLs) have specialized secretory lysosomes or lysosome-related organelles that store defined repertoires of proteins. In CTLs, lysosome secretion at the immunological synapse delivers lytic granules to effect target cell apoptosis (Stinchcombe et al., 2004). Exocytosis of 'conventional' lysosomes in response to a transient increase in intracellular calcium ($[Ca^{2+}]_i$) was first observed during cell invasion of *Trypanosoma cruzi*: binding of the parasite to the cell membrane triggers calcium influx and subsequent fusion of lysosomes with the region of the plasma membrane that surrounds the invading parasite (Andrews, 2002). Lysosome exocytosis has since emerged as an important mechanism in diverse paradigms such as membrane repair after exposure to calcium ionophores and pore-forming toxins (Divangahi et al., 2009; Jaiswal et al., 2002; Rodríguez et al., 1997) and for propagating the Ca^{2+} wave in astrocytes to modulate synaptic transmission (Li et al., 2008; Zhang et al., 2007). Molecular machinery implicated in calcium-induced lysosome exocytosis include the lysosomal calcium sensor synaptotagmin

VII, the v-SNARE VAMP7 and t-SNAREs SNAP23, syntaxin 2 and syntaxin 4 (Rao et al., 2004).

Although lysosomes have been identified as key organelles in membrane repair, studies show that other compartments distinct from lysosomes may also participate in wound healing (Divangahi et al., 2009; McNeil and Kirchhausen, 2005; Meldolesi, 2003). Rapid resealing of torn membranes is a ubiquitous, highly conserved response crucial for cell survival (McNeil and Kirchhausen, 2005). This is especially important for barrier epithelia that directly interact with pathogens and toxins, which can cause membrane lesions, necessitating rapid wound healing. However, whether lysosomes participate in membrane repair in polarized epithelia has not yet been examined. Here, we investigated lysosome exocytosis in polarized Madin-Darby canine kidney (MDCK) cells in response to pore-forming toxins and calcium ionophores. Our results demonstrate that (i) localized increase in $[Ca^{2+}]_i$ induces lysosomes to fuse exclusively with the basolateral membrane in MDCK cells; (ii) this polarized exocytosis is predicated on the actin cytoskeleton, membrane cholesterol and the basolateral localization of syntaxin 4; and (iii) the AP-1 clathrin adaptor specifies polarity of fusion by accurately targeting syntaxin 4 to the basolateral domain. These studies have implications for understanding lysosome

function in epithelial physiology and pathology, wound healing and immunity.

Results and Discussion

Calcium-induced lysosome exocytosis occurs preferentially at the basolateral membrane in polarized MDCK cells

Because calcium-induced lysosome exocytosis has never been documented in differentiated epithelial monolayers, we first established the polarity and extent of this process. Cells on Transwell filters were exposed to the calcium ionophore ionomycin (5 or 10 μ M) either in the apical, basolateral or both media. At these concentrations, ionomycin is not cytotoxic (supplementary material Fig. S1) and does not alter the trans-epithelial electrical resistance of the monolayer (supplementary material Table S1). In all cases, \sim 4-6% of total cellular β -hexosaminidase (β -hex) activity was present in the basolateral medium after ionomycin exposure (Fig. 1A). Enzyme activity in the apical medium was comparable to untreated cells. Ionomycin induced the appearance of lysosome-associated membrane protein LAMP2 exclusively on the basolateral surface (Fig. 1B), suggesting that lysosomes fuse predominantly with the basolateral membrane in polarized MDCK cells. Thrombin and bombesin, which mobilize intracellular calcium stores, and the pore-forming toxin streptolysin O (SLO) also induced lysosome exocytosis at the basolateral membrane (Fig. 1C; supplementary material Fig. S2). This exclusive basolateral fusion in response to increased $[Ca^{2+}]_i$ by various stimuli is supported by data showing that *Trypanozoma cruzi* enters MDCK monolayers only from the basolateral surface (Schenkman et al., 1988). Polarized exocytosis could reflect the asymmetric distribution of lysosomes and/or exocytic machinery at the basolateral surface or the existence of mechanisms that prevent apical exocytosis. Using apical and basolateral markers and ZO-1 to demarcate the tight junction, we observed that LAMP2-containing lysosomes in MDCK cells were distributed uniformly throughout the cell volume (supplementary material Fig. S3), suggesting that polarized exocytosis is not due to preferential basolateral docking of lysosomes. We then investigated mechanisms that could restrict lysosome fusion to the basolateral surface.

Polarity of lysosome exocytosis is lost upon actin depolymerization

Exocytic fusion with the plasma membrane requires remodeling of the actin cytoskeleton and actin depolymerization increases calcium-induced lysosome exocytosis in non-polarized cells (Rodríguez et al., 1999). In polarized epithelia, actin spatially restricts exocytosis by two mechanisms: one, cortical actin acts as a fusion barrier at the apical membrane (Ehre et al., 2005; Muallem et al., 1995); and two, an intact actin cytoskeleton is required to maintain syntaxin 4 clusters at the basolateral membrane (Low et al., 2006). In MDCK cells, the actin depolymerizing drug cytochalasin D caused lysosomes to fuse apically in response to ionomycin (Fig. 2A,B), whereas cytochalasin D alone had no effect on exocytosis (supplementary material Fig. S4). Immunofluorescence analysis confirmed that cytochalasin D disrupted actin filaments and dispersed syntaxin 4, but did not alter LAMP2 distribution (supplementary material Fig. S5). On the other hand, depolymerization of microtubules by treating the cells with nocodazole also disrupted syntaxin 4 clusters (supplementary

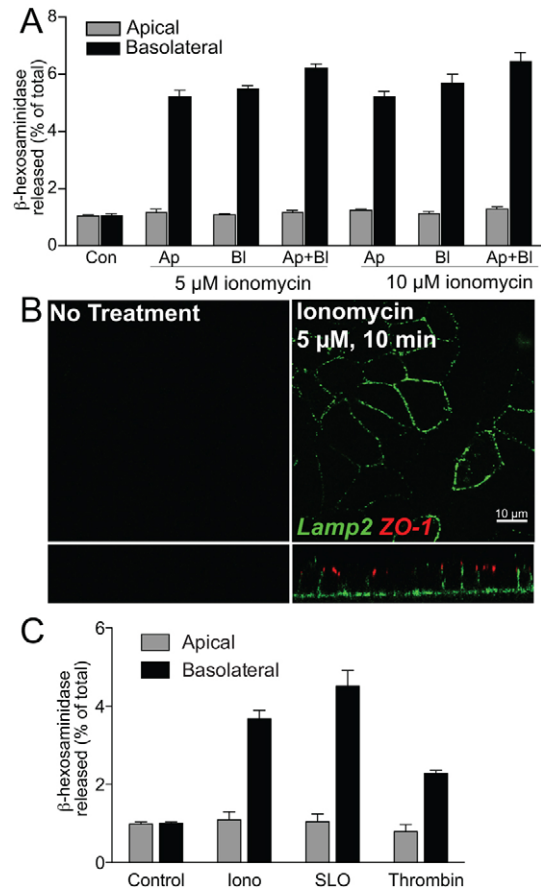


Fig. 1. Lysosomes fuse with the basolateral membrane in response to increased $[Ca^{2+}]_i$. (A) β -hex activity in apical (Ap) or basolateral (BI) media of polarized MDCK cells. Ionomycin (5 μ M or 10 μ M) was added to either or both compartments for 10 minutes and β -hex activity measured. Con, control untreated cells. (B) Appearance of LAMP2 (green) on the cell surface after ionomycin treatment. Confocal *xy* and *xz* sections are shown. The tight junction marker ZO-1 is in red. (C) Comparison of β -hex release after 10 minutes of incubation with 5 μ M ionomycin (Iono), 150 ng/ml streptolysin O (SLO) or 1 U/ml thrombin.

material Fig. S5), but did not alter the polarity of lysosome exocytosis (Fig. 2A,B). These data are in agreement with studies in non-polarized cells, where actin depolymerization increased and microtubule disruption had no effect on lysosome exocytosis (Jaiswal et al., 2002; Laulagnier et al., 2011). This is because microtubule-based motors are responsible for long-range movements that transport newly synthesized material and organelles such as lysosomes to specific locations within the cell, but do not participate in exocytosis (Caviston et al., 2011). Moreover, since \sim 5% of total cellular β -hex is released upon exocytosis in MDCK cells, only lysosomes already pre-docked near the plasma membrane likely exocytose in response to ionomycin, as has been demonstrated by Jaiswal and colleagues (Jaiswal et al., 2002). Therefore, microtubules are critical for positioning lysosomes near the plasma membrane, whereas cortical actin is a barrier to exocytosis: although both cytochalasin D and nocodazole dispersed syntaxin 4 to the apical membrane, only cytochalasin D induced apical lysosome fusion, presumably by increasing accessibility to the plasma membrane.

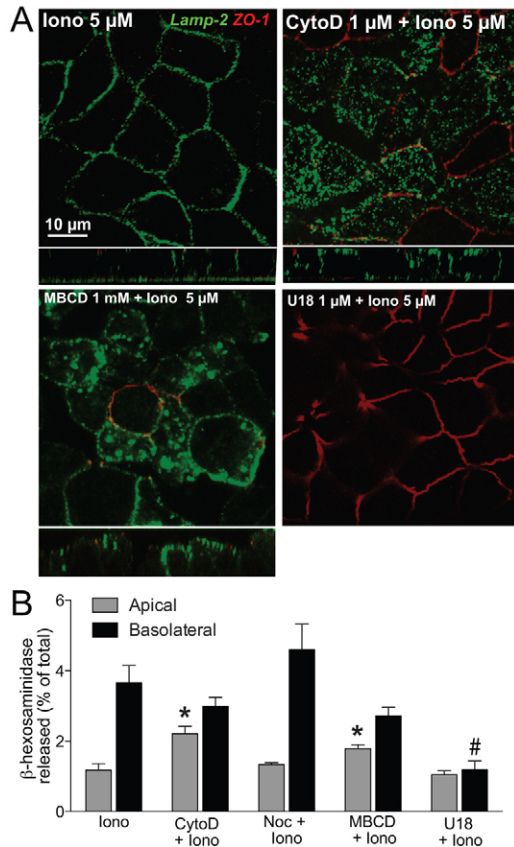


Fig. 2. Actin depolymerization and cholesterol extraction induce apical lysosome fusion. (A,B) MDCK cells were treated with nocodazole (Noc) to destabilize microtubules, cytochalasin D (CytoD) to depolymerize actin filaments, methyl- β -cyclodextrin (MBCD) to extract membrane cholesterol or U18666A (U18) to trap cholesterol in lysosomes prior to ionomycin (Iono) treatment. (A) Surface labeling for LAMP2 (green) and ZO-1 (red). (B) β -hex activity in apical and basolateral media. * $P < 0.01$ compared with apical β -hex release from cells treated with ionomycin alone; # $P < 0.01$, compared with basolateral release from cells treated with ionomycin alone.

Lysosome exocytosis is sensitive to membrane cholesterol levels

Cholesterol, an essential lipid in mammalian cells, participates in membrane biogenesis, modulates protein function and drives organelle traffic. In MDCK cells, extraction of membrane cholesterol by methyl- β -cyclodextrin (MBCD) before ionomycin exposure caused apical lysosome exocytosis (Fig. 2A,B), whereas MBCD alone had no effect on exocytosis (supplementary material Fig. S4). In contrast, U18666A, a hydrophobic amine that causes cholesterol accumulation in late endosomes and lysosomes (Huynh et al., 2008), completely inhibited ionomycin-induced lysosome exocytosis (Fig. 2A,B). Neither MBCD nor U18666A altered LAMP2 or actin distribution; however, MBCD dispersed syntaxin 4 clusters apically (supplementary material Fig. S6), in agreement with data from endothelial cells (Predescu et al., 2005). Recent reports show that in fibroblasts and cardiomyocytes, cholesterol depletion by cyclodextrins increases ionomycin-induced lysosome secretion (Chen et al., 2010; Hissa et al., 2012). Conversely, cholesterol accumulation in late endosomes and lysosomes paralyzes late endocytic traffic by a variety of mechanisms including inhibition of rabGTPases, which can interfere with the

recruitment of molecular motors to lysosomes, and thus reduce the population of pre-docked lysosomes at the plasma membrane, and abnormal sequestration of SNARE proteins, which would interfere with the fusion process (Fraldi et al., 2010; Lebrand et al., 2002).

The AP-1 clathrin adaptor complex regulates the basolateral exocytosis of lysosomes

Lysosomal membrane proteins LAMP1 and LAMP2 are transported from the trans-Golgi network to lysosomes via clathrin-coated transport intermediates. LAMPs contain C-terminal tyrosine-based sorting signals (YXX Φ), which bind the medium (μ) subunits of clathrin-adaptors AP-1, AP-2, AP-3 and AP-4 (Ohno et al., 1998). In polarized MDCK cells, newly synthesized LAMP2 is first transported to the basolateral membrane, endocytosed via clathrin and AP-2 and then sorted to lysosomes (Nabi et al., 1991). We have recently shown that clathrin regulates basolateral polarity (Deborde et al., 2008); this regulation is mediated by AP-1B (Fölsch et al., 1999; Gonzalez and Rodriguez-Boulan, 2009; Gravotta et al., 2007) and AP-1A (Carvajal-Gonzalez et al., 2012; Gravotta et al., 2012). The ubiquitously expressed AP-1A and the epithelial-specific AP-1B have identical γ , β 1, σ 1 subunits, but differ in their medium (μ) subunits, which are 80% identical: AP-1A has μ 1A, whereas AP-1B has μ 1B (Ohno et al., 1999). Pertinently, μ 1B was shown to participate in membrane targeting and lysosomal transport of LAMP1 in some studies (Höning et al., 1996; Sugimoto et al., 2002) but not in others (Karlsson and Carlsson, 1998; Laulagnier et al., 2011; Tazeh et al., 2009).

To investigate whether AP-1 participates in LAMP trafficking or lysosome exocytosis, we generated stable lines of MDCK cells lacking μ 1A [μ 1AKD (Carvajal-Gonzalez et al., 2012)] or μ 1B [μ 1BKD (Gravotta et al., 2007); supplementary material Fig. S7A]. Although LAMP2 levels in μ 1BKD cells were ~17% higher compared to wild-type and μ 1AKD cells (supplementary material Fig. S7B,C), we did not detect LAMP2 on the cell surface (Fig. 3A) or differences in the intracellular distribution of LAMP2 in μ 1AKD or μ 1BKD cells, compared to wild-type MDCK (supplementary material Fig. S3). Ionomycin induced the appearance of LAMP2 on both the apical and basolateral membranes of μ 1AKD and μ 1BKD cells, in contrast to its basolateral localization in wild-type cells (Fig. 3A). Quantification of surface LAMP2 signal showed that in wild-type cells, almost all the fluorescence was below ZO-1 (basolateral) whereas μ 1AKD and μ 1BKD cells showed two bumps in LAMP2 fluorescence, one above ZO-1 (apical) and the other below, indicating that loss of either μ 1 subunit resulted in non-polar exocytosis (Fig. 3B). This loss of polarity was confirmed by the non-polar secretion of β -hex in μ 1AKD, μ 1BKD and μ 1ABKD double knockdown cells compared to basolateral secretion in wild-type cells (Fig. 3C). Reconstitution of μ 1B activity in μ 1BKD cells (Diaz et al., 2009) restored basolateral polarity of lysosome exocytosis (Fig. 3D). Taken together, these data suggest that AP-1 regulates the basolateral fusion of lysosomes. Both AP-1A and AP-1B have been shown to mediate basolateral protein trafficking in polarized epithelia: whereas AP-1A promotes exit of basolateral proteins from the trans-Golgi network (TGN), AP-1B sorts proteins at the common recycling endosome (CRE) (Carvajal-Gonzalez et al., 2012; Gravotta et al., 2012; Gravotta et al., 2007). However, protein trafficking from the TGN and endosomes occurs along a continuum and these organelles cooperate to sort membrane

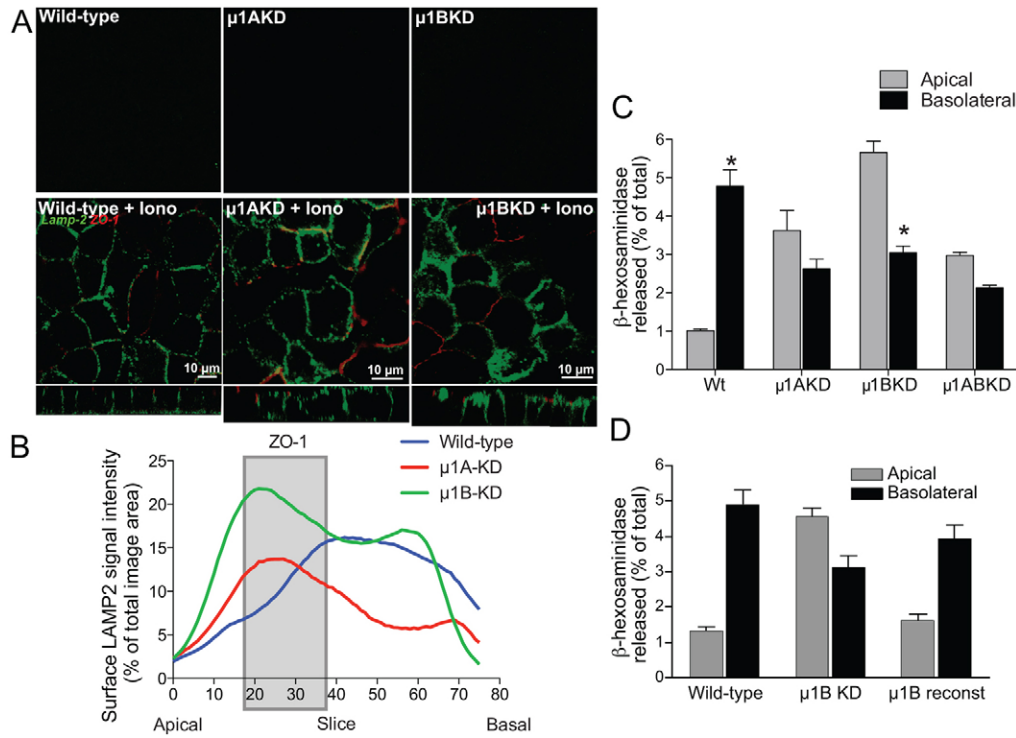


Fig. 3. The AP-1 adaptor is a crucial regulator of polarized lysosome exocytosis. (A) LAMP2 on the surface of wild-type MDCK cells or cells lacking μ 1A (μ 1AKD) or μ 1B (μ 1BKD) after ionomycin (Iono) treatment. (B) Quantification of surface LAMP2 fluorescence (expressed as a percentage of total fluorescence) in each confocal slice, numbered starting from the top of the monolayer. The gray bar indicates slices where ZO-1 is found and demarcates the apical and basolateral domains. (C) β -hex release from wild-type (wt), μ 1AKD, μ 1BKD or μ 1ABKD double knockdown cells. * $P < 0.001$, compared with corresponding apical values. (D) β -hex release from wild-type, μ 1BKD and μ 1BKD cells with reconstituted μ 1B.

proteins to their correct destinations: for instance, in the absence of AP-1A, AP-1B has been shown to relocate to the TGN and mediate cargo exit (Carvajal-Gonzalez et al., 2012; Gravotta et al., 2012; Gravotta et al., 2007). In the case of lysosome exocytosis, it is likely that AP-1A and AP-1B cooperate in the basolateral targeting of a protein critical for this process. What could be the putative AP-1 cargo that specifies basolateral exocytosis?

The t-SNARE syntaxin 4 interacts with the AP-1 adaptor complex and directs lysosome fusion

Exocytosis of lysosomes and secretory granules requires interactions between lysosomal v-SNARE VAMP7 and t-SNAREs syntaxin 4 and SNAP-23 (Rao et al., 2004). In polarized MDCK cells and in the kidney *in vivo*, syntaxin 4 localizes to the basolateral membrane (Low et al., 1996). Because AP-1 sorts basolateral proteins (Fölsch et al., 1999; Gravotta et al., 2012; Gravotta et al., 2007), we asked whether syntaxin 4 is an AP-1 cargo. In wild-type, μ 1AKD and μ 1BKD MDCK cells, endogenous syntaxin 4 coimmunoprecipitated with γ -adaptin (Fig. 4A). Our data also show that syntaxin 4 is a specific cargo for AP-1 because syntaxin 2, a t-SNARE implicated in lysosome exocytosis in platelets (Chen et al., 2000), did not coimmunoprecipitate with γ -adaptin (Fig. 4A). Immunofluorescence analysis showed that endogenous syntaxin 4 is expressed at low levels in wild-type MDCK cells and localizes mainly at or below the tight junction (Fig. 4B). In μ 1AKD and μ 1BKD cells, we observed a few cells with syntaxin 4 at or near the apical membrane (Fig. 4B). To further clarify this, we generated stable cell lines of wild-type, μ 1AKD and μ 1BKD cells expressing myc-tagged syntaxin 4. Exogenous syntaxin 4 was found exclusively at the basolateral membrane in wild-type MDCK cells as has been previously reported (Low et al., 1996); in μ 1AKD and μ 1BKD cells, syntaxin 4-myc was found on both the apical and basolateral

membranes (supplementary material Fig. S8). However, we were unable to detect a direct interaction between syntaxin 4 and μ 1A or μ 1B by yeast two-hybrid analysis, likely due to low binding affinity or the participation of a linker protein. While our studies were underway, it was reported that syntaxin 4 is nonpolar in a porcine proximal tubule cell line, LLCPK1, which lacks μ 1B (Reales et al., 2011). To further elucidate how AP-1 and syntaxin function in polarized lysosome secretion, we used shRNA-mediated knockdown of endogenous syntaxin 4 (Fig. 4C) (Torres et al., 2011). In cells treated with the scrambled shRNA, ionomycin induced basolateral β -hex release in wild-type MDCK, and nonpolar secretion in μ 1AKD and μ 1BKD cells. However, syntaxin 4 depletion inhibited basolateral lysosome exocytosis in wild-type cells, and both apical and basolateral exocytosis in μ 1AKD and μ 1BKD cells in response to ionomycin (Fig. 4D).

Taken together, our data and recently published work (Carvajal-Gonzalez et al., 2012; Gravotta et al., 2012; Gravotta et al., 2007) support a model where, in wild-type MDCK cells, newly synthesized syntaxin 4 is transported out of the TGN by AP-1A and sorted to the basolateral membrane by endosomal AP-1B. As has been demonstrated for other basolateral proteins, μ 1B can likely compensate for the absence of μ 1A (μ 1AKD cells) by mediating the exit of syntaxin 4 from the TGN and promoting increased trafficking to the CRE (Carvajal-Gonzalez et al., 2012; Gravotta et al., 2012; Gravotta et al., 2007). At the CRE, μ 1B is required to sort syntaxin 4 to the basolateral membrane and in μ 1BKD cells, syntaxin 4 is rerouted to the apical membrane, similar to other basolateral proteins (Carvajal-Gonzalez et al., 2012; Gravotta et al., 2012; Gravotta et al., 2007). In μ 1AKD cells, high levels of syntaxin 4 in the CRE could overwhelm the μ 1B sorting apparatus, which would also lead to mis-localization of syntaxin 4 to the apical surface. At the membrane, syntaxin 4 clusters are stabilized by association with the actin cytoskeleton and cholesterol-enriched microdomains.

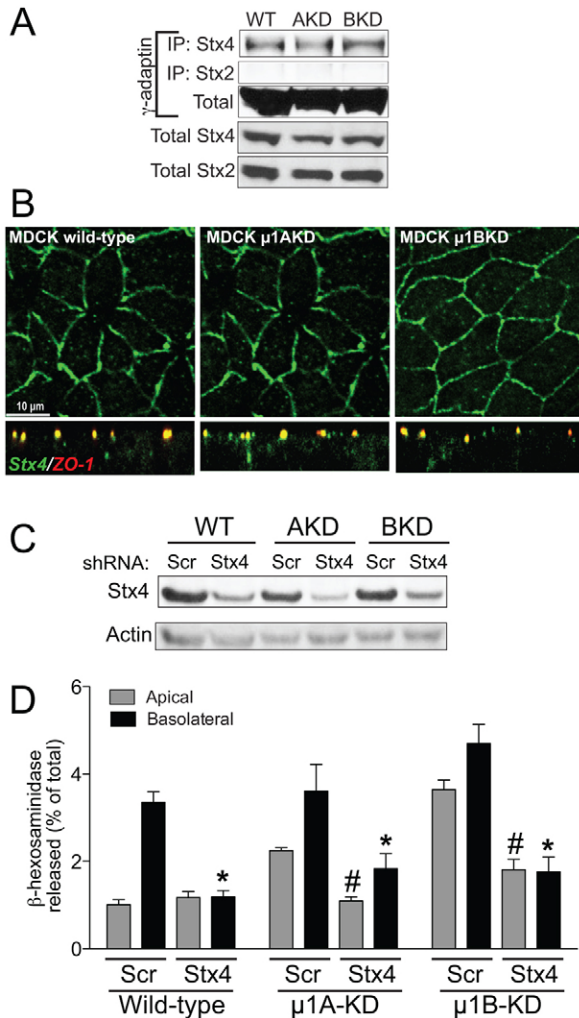


Fig. 4. Syntaxis 4 interacts with AP-1 and is essential for lysosome exocytosis. (A) Endogenous syntaxis 4 (Stx4) or syntaxis 2 (Stx2) were immunoprecipitated from wild-type (WT), μ 1AKD and μ 1BKD MDCK cell lysates (300 μ g each) and immunoblotted for γ -adaptin. Inputs: 30 μ g each of total lysates probed for γ -adaptin, syntaxis 4 and syntaxis 2. (B) Immunostaining for endogenous syntaxis 4 (green) in wild-type, μ 1AKD and μ 1BKD cells. ZO-1 is in red. (C) Representative western blot showing shRNA-mediated knockdown of endogenous syntaxis 4 in wild-type, μ 1AKD and μ 1BKD cells; Scr, scrambled sequence. Actin was the loading control. (D) β -hex release from cells transfected with either scrambled or syntaxis 4 shRNA constructs. * $P < 0.05$, compared with basolateral release from corresponding Scr cells; # $P < 0.05$ compared with apical release from corresponding Scr cells.

Conditions that cause apical redistribution of syntaxis 4 (actin depolymerization, membrane cholesterol extraction, absence of μ 1A or μ 1B) lead to nonpolar calcium-induced lysosome secretion. Apical exocytosis of lysosomes has been documented *in vivo* in hepatocytes (Gross et al., 1989) and the proximal tubule of the kidney (Fujita et al., 1998), both of which lack μ 1B (Ohno et al., 1999; Schreiner et al., 2010). Indeed, syntaxis 4 is apical in hepatocytes (Fujita et al., 1998) and in LLC-PK1 cells (Reales et al., 2011), providing further support for the role of μ 1B in restricting syntaxis 4 to the basolateral membrane and regulating the polarity of lysosome fusion.

How then does apical wound healing occur in the vast majority of epithelia that do express AP-1B? Recent reports have implicated galectin-7 in primary cilia of kidney epithelia and rab11-dependent expulsion of microvilli in intestinal epithelia (Los et al., 2011; Rondanino et al., 2011) in apical membrane repair. Apart from wound healing, lysosome exocytosis can be exploited for treating lysosome storage disorders by promoting cellular clearance of accumulated debris (Chen et al., 2010; Medina et al., 2011). Insight into the molecular mechanisms involved in polarized lysosome exocytosis as detailed in this study will aid our understanding of these diverse processes central to both epithelial physiology and pathology.

Materials and Methods

Cells

MDCK Type II cells were cultured in DMEM (Cellgro) with 5% FBS (Gemini Biosciences) on semi-permeable Transwell® filters (BD Biosciences). Cells were plated at confluence ($\sim 250,000/\text{cm}^2$) for four days after which they are fully polarized. Stable cell lines lacking μ 1A or μ 1B, μ 1AB double knockdown cells and reconstitution of μ 1B into μ 1BKD cells were generated and maintained as described elsewhere (Carvajal-Gonzalez et al., 2012; Diaz et al., 2009; Gravotta et al., 2012; Gravotta et al., 2007).

Lysosome exocytosis

Cells were rinsed in recording medium (HBSS with 4.5 g/l glucose, 20 mM HEPES) and incubated with ionomycin (Sigma) for 10 minutes at 37°C. Alternatively, streptolysin O (SLO) was used to trigger exocytosis (Idone et al., 2008). SLO [150 ng/ml, (Abcam)] was thiol-activated and bound to cells in Ca^{2+} -free medium for 5 minutes at 4°C and pore formation was induced by replacing the medium with fresh recording medium at 37°C for 10 minutes. Filters were immediately transferred to ice and analyzed for surface LAMP2 or β -hex activity (see below). Other drugs used were (Sigma): 1 U/ml thrombin, cytochalasin D (1 μ M, 1 hour), nocodazole (10 μ M, 1 hour), U18666A (1 μ M, 16 hours), methyl- β -cyclodextrin (1 mM, 1 hour).

Detection of cell-surface LAMP2

Cells on ice were incubated with a monoclonal antibody to the luminal domain of LAMP2 (Nabi et al., 1991) in recording medium+1% BSA for 30 minutes at 4°C, fixed with 2% paraformaldehyde, permeabilized and stained for ZO-1 and Alexa-Fluor-conjugated secondary antibodies (Invitrogen) for 30 minutes. Cells were imaged by confocal microscopy (Leica SP2 or Andor Revolution XD) using a 63 \times 1.4 NA or 60 \times , 1.4 NA oil objective. For each set of experiments, the laser power, voltage and offset were identical for a given fluorophore. Z-stacks were acquired for six different fields, ~ 150 cells/field, with fields that showed as little variance in the height of the cells in Z as possible. To quantify surface LAMP2 fluorescence, images from each cell line with corresponding Z-stacks (same number of slices) were analyzed using Andor IQ2 software. The percent area occupied by the LAMP2 signal in each Z slice was calculated using the Analysis feature in IQ2, and plotted as a function of confocal slice. ZO-1 staining was used to demarcate the apical and basolateral domains of the cell.

Measurement of β -hex activity

After drug treatments, apical and basolateral media were collected, centrifuged at 100 g for 5 minutes to pellet dead cells and 10,000 \times g for 5 minutes to pellet debris. Cells were lysed in 0.5 ml PBS+1% NP-40 for total β -hex activity. To measure enzyme activity, 350 μ l of supernatant was incubated for 20 minutes with 50 μ l of 6 mM 4-methyl-umbelliferyl-N-acetyl- β -D-glucosaminide (Sigma) in sodium citrate-phosphate buffer, pH 4.5 (Rodríguez et al., 1999). Fluorescence was measured after stopping the reaction with 100 μ l 2 M Na_2CO_3 , 1.1 M glycine (365 nm excitation, 450 nm emission, SpectraMax Gemini microplate reader (Molecular Devices)). Cell extracts were diluted 1:50 before assay for total cellular β -hex activity.

Immunofluorescence staining

To determine the polarity of endogenous syntaxis 4, wild-type, μ 1AKD and μ 1BKD MDCK cells were fixed, permeabilized and stained with mouse α -syntaxis 4 (BD Biosciences, 1:400) and rat α -ZO-1, and Alexa-conjugated secondary antibodies and imaged by spinning disk confocal microscopy (Andor Revolution XD) using a 60 \times 1.4 NA oil objective. Drug treated cells were labeled with rhodamine-phalloidin (Cytoskeleton, Inc.) to visualize the actin cytoskeleton or stained with mouse α -beta-tubulin (Sigma) to visualize microtubules.

Immunoprecipitation and immunoblotting

300 µg of total protein from wild-type, µ1AKD and µ1BKD cell lysates were immunoprecipitated with 5 µg of α-syntaxin 4 or α-syntaxin 2 (Abcam) using the Dynabead Protein G immunoprecipitation kit (Invitrogen) and probed for γ-adaptin (Sigma) after SDS-PAGE. Total cell lysates (30 µg) were subjected to SDS-PAGE and probed for γ-adaptin, syntaxin 4 and syntaxin 2.

RNA interference

Syntaxin 4 shRNA (5'-CCGGATTGAGAAGAACATC-3' (Torres et al., 2011) and a scrambled sequence were subcloned into a pGFP-B-RS vector (Origene). Cells were transfected by Amaxa nucleofection (Lonza) and plated on to Transwell filters for experiments. Syntaxin 4 knockdown was confirmed by immunoblotting using a rabbit polyclonal antibody (Sigma).

Statistical analysis

Data were analyzed by a two-tailed *t*-test or one-way ANOVA with the Bonferroni post-test (GraphPad Prism). Unless otherwise stated, data are presented as mean ± s.e.m. of more than six independent measurements.

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Supplementary material available online at

<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.109421/-DC1>

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Figure S1. Lactate dehydrogenase release after ionomycin treatment
 Lactate dehydrogenase activity (Cayman Chemical) was measured in the apical and basolateral media of filter-grown MDCK cells after a 10 minute treatment with 0, 5 or 10 μ M ionomycin.

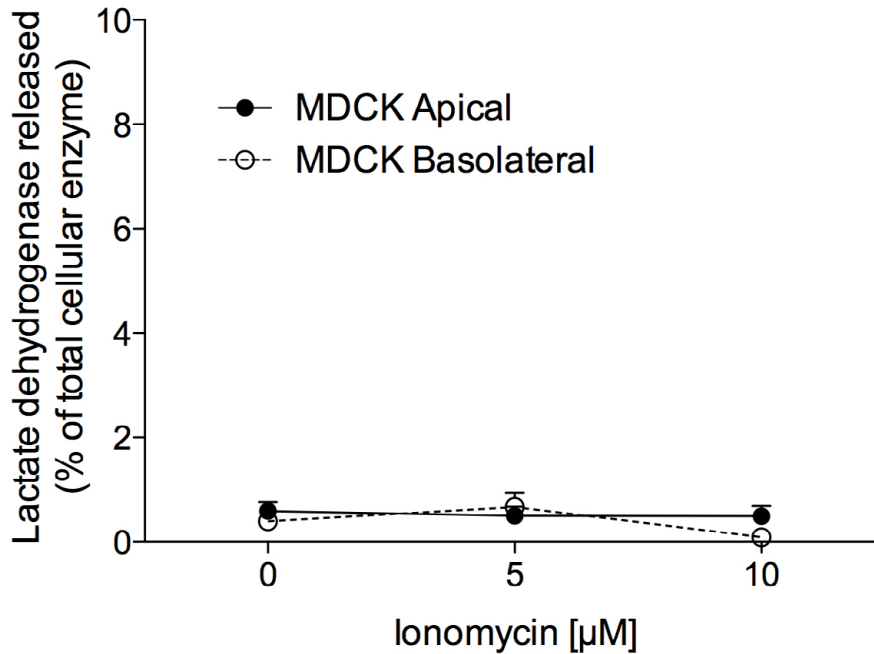
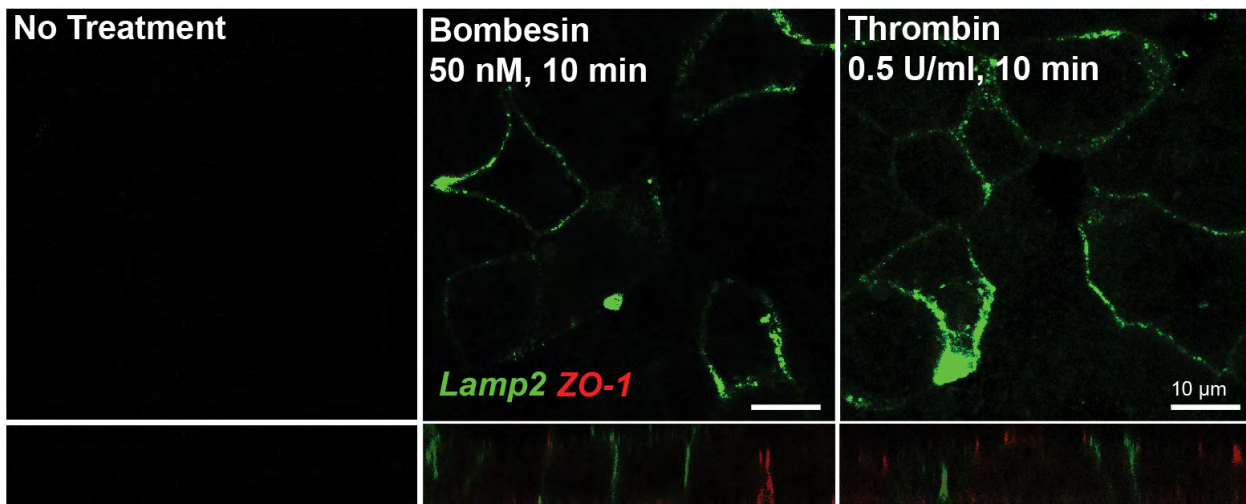


Figure S2. Lysosome exocytosis in MDCK cells induced by thrombin and bombesin.
 MDCK cells were treated with either bombesin or thrombin (Sigma), labeled on ice with the LAMP-2 antibody, fixed and stained for the tight junction marker ZO-1 and the relevant secondary antibodies as described in the main text.



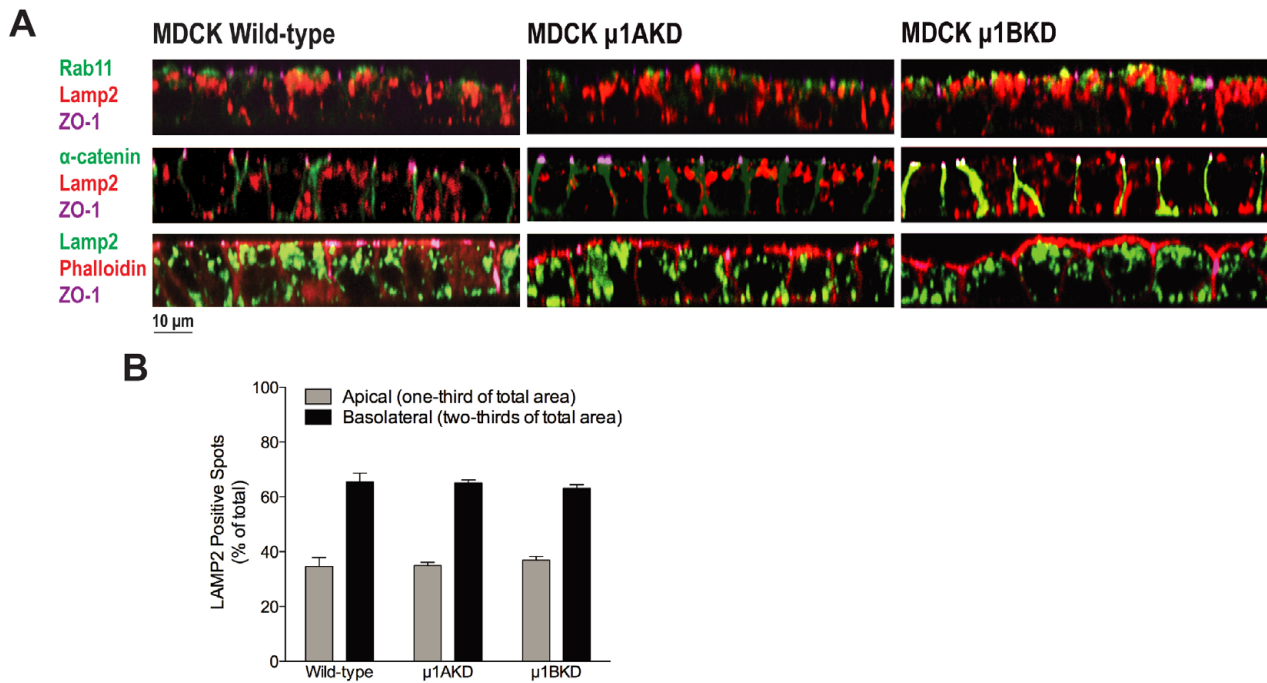


Figure S3. Distribution of lysosomes in MDCK cells.

A, Confocal x-z sections of LAMP2 staining in wild-type and μ 1BKD MDCK cells with apical (rab11) and basolateral (α -catenin) markers or with phalloidin, which stains both the apical and basolateral membranes. In the top two rows, the apical recycling endosome marker rab11 and the basolateral α -catenin are in green, LAMP2 is in red and the tight junction protein ZO-1 in purple. In the bottom row, LAMP2 is in green and phalloidin is in red. Antibodies used were: rabbit polyclonal to rab11 (Invitrogen, 1:500), mouse monoclonal to LAMP2 (Abd Serotec, 1:1000), rabbit anti- α -catenin (Sigma, 1:1000) and rat anti-ZO-1. Using the Andor Revolution XD spinning disk confocal microscope, Z-stacks were acquired for 6 different fields, approximately 150 cells/field, with fields that showed as little variance in the height of the cells in Z as possible. **B**, Quantification of LAMP2 fluorescence above (apical) and below (basolateral) the tight junction (ZO-1) in wild-type, μ 1AKD and μ 1BKD MDCK cells. Imaris software was used to identify LAMP2 positive vesicles (Spots function) and the apical and basolateral surfaces were demarcated by using the Surfaces function to identify the band of ZO-1 running along the apical border of each cell. The number of LAMP2 positive spots occurring above and below ZO-1 were quantified.

Xu et al, Supplementary Fig S4

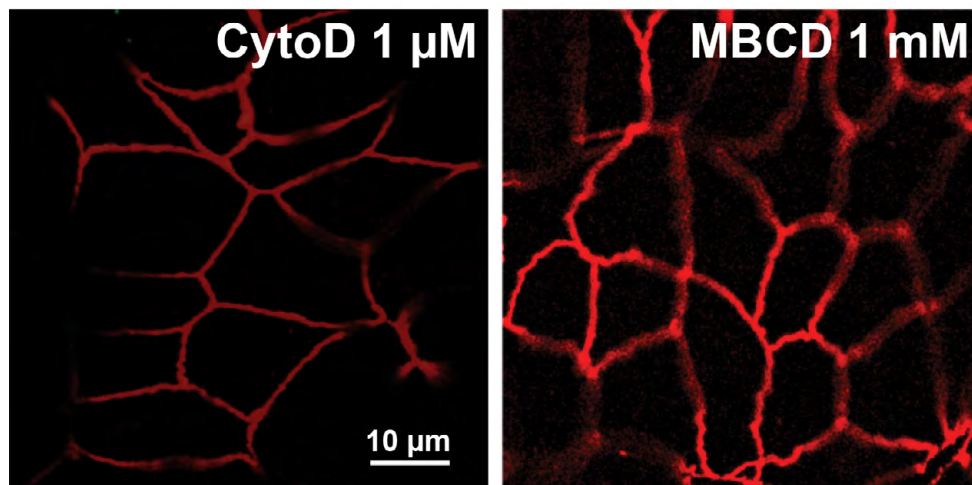


Figure S4. Drug treatments and lysosome exocytosis

Surface LAMP2 staining (green) in MDCK cells treated with the actin depolymerizing drug cytochalasin D (CytoD, 1 μ M, for 1 h) or with methyl-beta-cyclodextrin (MBCD, 1 mM for 1 h) shows that these treatments alone did not induce lysosome exocytosis. ZO-1 is in red.

Figure S5. Actin and microtubule disrupting drugs

Polarized MDCK cells were treated with cytochalasin D (1 μ M, 1 h) or with nocodazole (10 μ M, 1 h) to disrupt actin and microtubules, respectively. Cells were fixed and stained with phalloidin to visualize the actin cytoskeleton and with antibodies to tubulin (Sigma) for microtubules, LAMP2 and syntaxin 4. Cells were imaged on the Andor Revolution XD spinning disk confocal microscope.

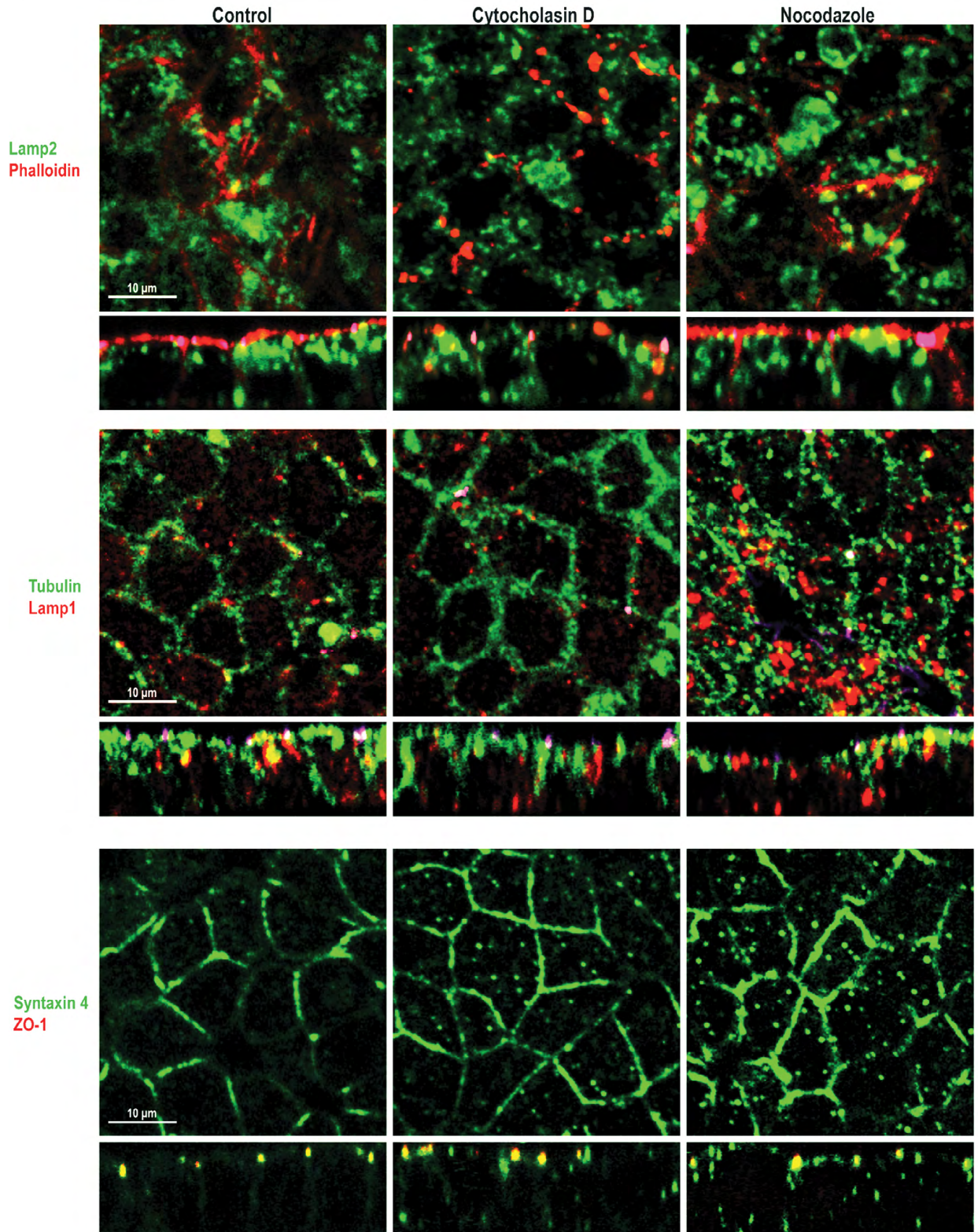
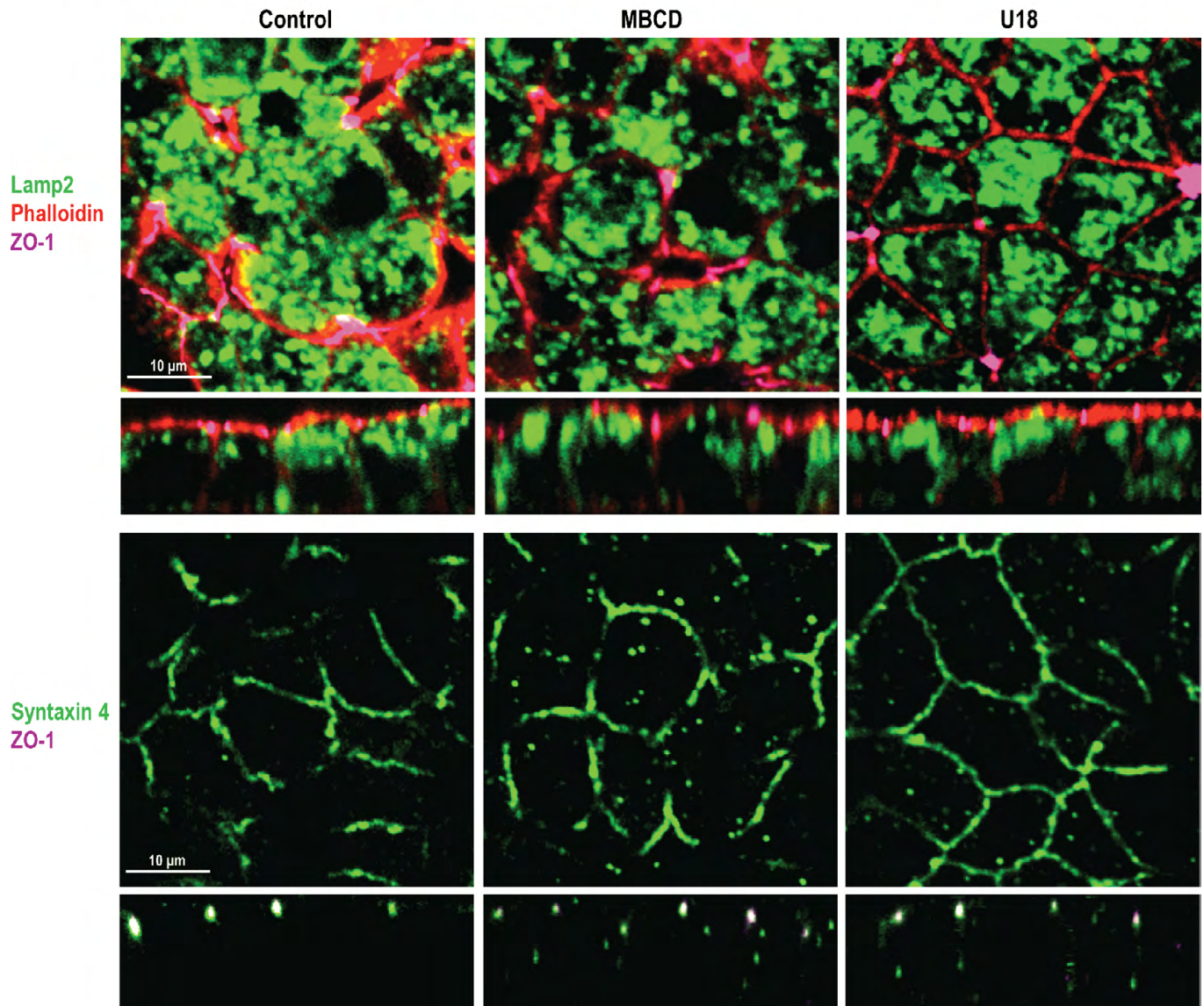


Figure S6. Methyl-beta-cyclodextrin and U18666A treatments

Polarized MDCK cells were treated with methyl-beta-cyclodextrin (MBCD, 1 mM, 1 h) to deplete membrane cholesterol or with U18666A (U18, 1 μ M, 16 h) to induce lysosomal cholesterol storage. Cells were fixed and stained with phalloidin to visualize the actin cytoskeleton and with antibodies to LAMP2 and syntaxin 4. Cells were imaged on the Andor Revolution XD spinning disk confocal microscope.



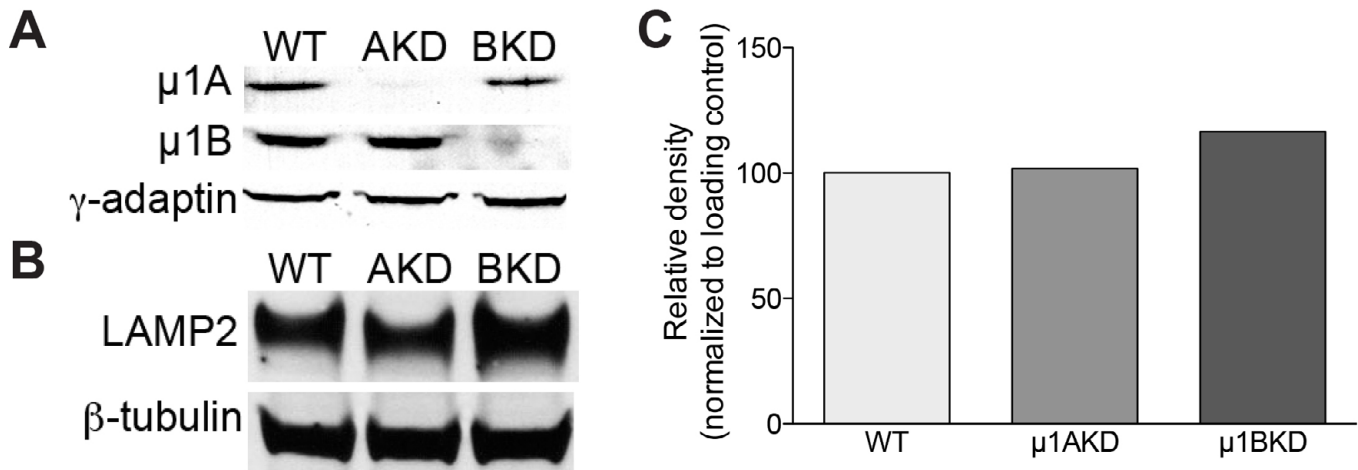


Figure S7. μ1A and μ1B expression in wild-type, μ1AKD and μ1BKD MDCK cells

A, μ1A, μ1B and γ-adaptin levels in wild-type (WT), μ1AKD (AKD) and μ1BKD (BKD) cell lysates were determined by immunoblotting with specific μ1A and μ1B antibodies (Gravotta et al, 2012).

B, Representative immunoblot showing total LAMP2 levels in wild-type, μ1AKD and μ1BKD cell lysates were determined by immunoblotting with anti-LAMP2 (ABD Serotec). **C**, Quantitation of LAMP2 bands normalized to loading control, mean of two independent experiments.

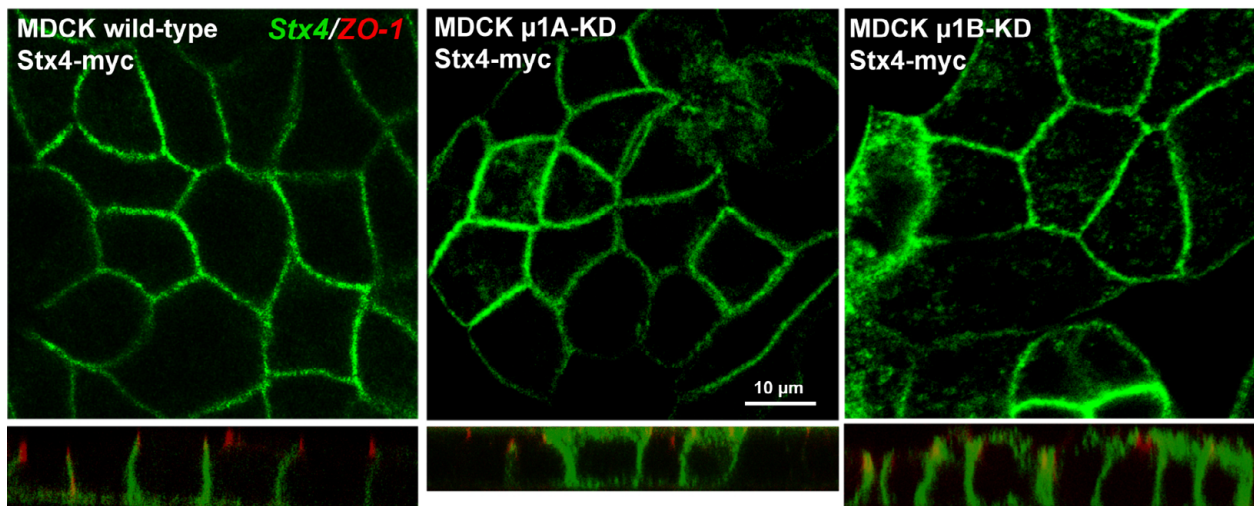


Figure S8. Localization of exogenously expressed syntaxin 4-myc in MDCK cells.

Wild-type, μ1AKD and μ1BKD cells were transfected with syntaxin 4-myc and stable cell lines were generated using zeocin. Polarized monolayers were fixed, permeabilized and stained with anti-syntaxin 4 (green) and ZO-1 (red).

Table S1. Trans-epithelial resistance (TER) of polarized MDCK cells cultured on Transwell filters before and immediately after treatment with ionomycin.

Ionomycin, 10 min	MDCK TER ($\Omega \cdot \text{cm}^2$)	
	Before	After
0 μM	114 \pm 11.5	113.7 \pm 7.8
5 μM	106.7 \pm 4.93	109.7 \pm 8.74
10 μM	111.7 \pm 11.55	106.0 \pm 5.29

TER was measured using the EVOM-2 epithelial volttohmmeter (World Precision Instruments). Mean \pm SD, n = 3 for each condition.