Drebrin E depletion in human intestinal epithelial cells mimics Rab8a loss of function

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Received July 18, 2013; Revised November 30, 2013; Accepted December 26, 2013

Intestinal epithelial cells are highly polarized and exhibit a complex architecture with a columnar shape and a specialized apical surface supporting microvilli organized in a brush border. These microvilli are rooted in a dense meshwork of acto-myosin called the terminal web. We have shown recently that Drebrin E, an F-actin-binding protein, is a key protein for the organization of the terminal web and the brush border. Drebrin E is also required for the columnar cell shape of Caco2 cells (human colonic cells). Here, we found that the subcellular localization of several apical markers including dipeptidyl peptidase IV (DPPIV) was strikingly modified in Drebrin E-depleted Caco2 cells. Instead of being mostly present at the apical surface, these proteins are accumulated in an enlarged subapical compartment. Using known intracellular markers, we show by both confocal and electron microscopy that this compartment is related to lysosomes. We also demonstrate that the enrichment of DPPIV in this compartment originates from apical endocytosis and that depletion of Rab8a induces an accumulation of apical proteins in a similar compartment. Consistent with this, the phenotype observed in Drebrin E knock-down Caco2 cells shares some features with a pathology called microvillar inclusion disease (MVID) involving both Myosin Vb and Rab8a. Taken together, these results suggest that Drebrin E redirects the apical recycling pathway in intestinal epithelial cells to the lysosomes, demonstrating that Drebrin E is a key regulator in apical trafficking in Caco2 cells.

INTRODUCTION

Intestinal epithelial cells are characterized by their columnar cell shape (1) and their apico-basal polarity that allows them to differentiate the plasma membrane into distinct apical and a basolateral domains essential for their function (2). For example, the apical membrane is involved in nutrient absorption and is characterized by the differentiation of the brush border, which is made up of microvilli increasing the contact surface area tremendously (1), facilitating nutrient uptake and enhancing the efficacy and the expression of digestive enzymes specific to the brush border, such as dipeptidyl peptidase IV (DPPIV) or sucrase–isomaltase (SI). The border between the apical and the basolateral domains is based on tight junctions that ensure the integrity of the epithelial tissue and the establishment of a selective barrier between the external and internal environment (3). In addition to tight junctions, the apico-basal polarity is also reflected by the polarized organization of the cytoskeleton and a tightly regulated network of intracellular trafficking.

The apical cytoskeleton is made of actin filaments extended from the microvilli to the dense F-actin meshwork of the subapical terminal web where they are rooted (4). The terminal web is also able to link with the tight junctions. Actin-binding proteins enriched in this domain are thus potent regulators of this specialized subapical organization. This complex cytoskeleton network is important for polarized intracellular trafficking (5),

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and in particular, the subapical terminal web regulates the spatial organization of apical exocytosis and endocytosis/recycling (6). Polarized trafficking must therefore require regulatory mechanisms that involve both the cytoskeleton and motor proteins (7). For example, mutations in the MYO VB gene are the main cause of a rare disease in newborns called the microvillar inclusion disease (MVID; OMIM 251850), leading to intestinal malabsorption (8). In particular, defects in MyoVb expression in human intestinal cells in vitro and in vivo are associated with a disruption of the apical membrane microvilli organization and the accumulation of apical proteins in intracellular compartments without affecting cell polarity (9,10). While MYO VB is the only mutated gene identified so far in MVID patients, some patients do not show any defects in this gene. Several other proteins, however, have been shown to either interact with MyoVb or induce a very similar phenotype when compromised. Among them are Rab8a, Rab11 (11,12) and Cdc42 (13). Rab8a conditional knock-out (cKO) mice, for example, show an enrichment of apical membrane proteins in a lysosomal compartment concomitant with a decrease in their total expression level. In addition, microvillus inclusions are present in the apical cytoplasm of 10% of enterocytes (14), reminiscent of a MVID phenotype. Rab8a acts as a linker between the transport vesicle and the motor protein MyoVb (11), and both proteins cooperate in the apical trafficking pathway.

Actin-binding proteins such as MyoVb that are enriched at the terminal web are thus potent regulators of the specialized subapical organization. Among them, Drebrin A and E isoforms are actin-binding proteins mainly described for their functions in neuronal morphogenesis (15). Drebrin proteins are involved in the regulation of neurite outgrowth, axonal differentiation and dendritic spines morphogenesis (16–18). Drebrin E is also expressed in stomach and kidney (19), but its function is still unknown in these organs.

Previously, we have shown that the Drebrin E isoform is also expressed in enterocytes and Caco2 cells and is enriched at the terminal web, while accumulating locally along the lateral membrane (20). In that study, we have uncovered a new function of Drebrin E in epithelial cell morphogenesis that is essential for epithelial cell compaction and elongation (20). As Drebrin E depletion disrupted the organization of apical microvilli and subapical F-actin terminal web (20), we investigated its role in apical protein transport and localization in Caco2 cells, a well-established in vitro model for enterocytes and studies of MVID (9). We showed that in Caco2 cells that were transiently depleted for Drebrin E, apical membrane protein expression is specifically decreased owing to a defect in their biosynthesis. Moreover, apical membrane proteins still present in Drebrin E knock-down (KD) cells accumulate in a compartment positive for both multi-vesicular body (CD63) and lysosomal (LAMP1, Cathepsin D) markers after their endocytosis from the apical membrane. This accumulation of apical membrane proteins in a compartment designated for degradation was also found in Rab8a KD Caco2 cells and is reminiscent of the Rab8a cKO phenotype. In addition, we observed a strong cytoplasmic redistribution of Drebrin E upon Rab8a KD in Caco2 cells and Rab8a KO in mouse. Taken together, these results demonstrate that both Rab8a and Drebrin E act as key proteins in the regulation of apical trafficking probably by affecting apical recycling.

RESULTS

Drebrin E depletion causes accumulation of apical membrane proteins in an intracellular compartment

We had previously shown that the transient depletion of Drebrin E in Caco2 cells leads to the disorganization of the subapical acto-myosin cytoskeleton without affecting apico-basal polarity (20). This subapical cytoskeleton is required for brush border maintenance and apical constriction during cell elongation. To characterize how Drebrin E depletion results in apical membrane disorganization, we asked whether the localization of apical markers, that are known to be associated with the intestinal brush border, is affected when Drebrin E is compromised. To this aim, we immunolabeled control (CT) and Drebrin knock-down (KD) Caco2 cells for several apical markers such as SI, DPP IV, intestinal alkaline phosphatase (IAP) and carcinoembryonic antigen (CEA). These markers showed a strong apical staining in CT cells whereas in Drebrin KD cells, they accumulated in large intracellular compartments (Fig. 1A and B, and data not shown). Moreover, this intracellular accumulation of apical markers in Drebrin KD cells correlated with a decrease in their apical staining when compared with Caco2 CT cells (Fig. 1A and B). However, we did not observe a significant change in the localization of several basolateral markers (E-cadherin, Ag525, Scribble and Transferrin receptor) [(20) and data not shown]. Finally, as we had previously shown, the actin cytoskeleton was strongly disorganized in Drebrin E KD cells with a decrease in the subapical staining (20). Nevertheless, no overlap was observed between the intracellular compartment positive for the apical markers and actin in Drebrin E KD cells (Fig. 1B).

To examine the ultrastructural morphology of the intracellular storage compartment induced by Drebrin E depletion, we performed transmission electron microscopy (TEM) on Caco2 cells. Consistent with our previous study (20), the apical domain was disorganized in Drebrin E KD cells producing only a few microvilli whereas elongation and compaction were defective (Fig. 1C and data not shown). In addition to these defects in morphogenesis, we observed large intracellular dense compartments mainly localized between the nucleus and the apical plasma membrane in Drebrin E KD cells (Fig. 1C). These large, dense intracellular compartments were of heterogeneous content with either amorphous material or membrane accumulations and were never observed in CT cells, indicating that they were specific to Drebrin E KD Caco2 cells (Fig. 1C).

We previously showed that the disorganization of the subapical cytoskeleton occurred between Day 3 (D3) and 5 (D5) after transfection in Drebrin E KD cells (20). To investigate when apical markers were first enriched in these large, dense intracellular organelles, we performed DPP IV immunolabeling at D2, D3, D4 and D5 on CT and Drebrin E KD cells. We found that intracellular organelles positive for DPP IV were already detected at D2, indicating that their formation was likely the consequence of an early defect in Drebrin E KD Caco2 cells (Supplementary Material, Fig. S1A and B for D3–D5, not shown for D2). When we checked for the presence of F-actin or phospho-myosin II, these two proteins were never accumulated in these structures (not shown), demonstrating that no brush border was present, as we could already observed by EM. Together, these results demonstrate that Drebrin E depletion results in an early
intracellular accumulation of apical membrane proteins in a large and heterogeneous compartment.

**Apical membrane proteins accumulate in a large degradation compartment in Drebrin E-depleted Caco2 cells**

To determine the identity of this large, dense intracellular compartment that results from Drebrin E depletion, we used markers for various intracellular compartments involved in the exocytic and endocytic pathways. We found a strong co-localization in this large intracellular compartment between the apical marker DPPIV, and LAMP1, a marker of the late endosomes/lysosomes, and CD63, a marker of multi-vesicular bodies, or Cathepsin D, a marker of lysosomes (Fig. 2B and C) by confocal and electron microscopy (Supplementary Material, Fig. S2A). Neither LAMP1 nor CD63 or Cathepsin D co-localized with DPPIV in CT cells, indicating that the co-localization was a direct consequence of Drebrin E depletion (Fig. 2B and C). To demonstrate that this enrichment in the degradation pathway after Drebrin E depletion was a general feature for apical membrane proteins, we used several apical markers (SI, DPPIV, CEA and IAP) and found that they all co-localized in these degradation organelles in Drebrin E KD cells (Fig. 2A for DPPIV and SI, and not shown for CEA and IAP). In addition, we did not detect any co-localization between apical membrane proteins and markers of trans-Golgi network (TGN46), early endosomes (EEA-1) or recycling endosomes (Rab11a), in either CT or Drebrin KD cells (Supplementary Material, Fig. S2B). These
Figure 2. Apical membrane proteins are relocated in an enlarged degradative compartment in Drebrin E knock-down Caco2 cells. (A) Subcellular co-localization of SI (green) and dipeptidyl peptidase IV (DPP IV—red) in Drebrin E knock-down (Dreb KD) but not in CT cells. Arrowheads, intracellular compartment positive for DPP IV and SI. Scale bar: 10 μm. (B) Dipeptidyl peptidase IV (DPP IV—red) subcellular localization in CT and Drebrin E knock-down (Dreb KD) cells. LAMP1 (late endosomal/lysosomal marker—green; upper panels) and CD63 (multi-vesicular body marker—green; bottom panels) co-localize with DPP IV in KD but not in CT cells. Arrowheads, intracellular compartment positive for DPP IV, LAMP1 and CD63. Scale bar: 10 μm. (C) Dipeptidyl peptidase IV (DPP IV—red) subcellular localization in CT and Drebrin E knock-down (Dreb KD) cells. LAMP1 (late endosomal/lysosomal marker—green) and Cathepsin D (lysosome marker—cyan) co-localize with DPP IV in KD but not in CT cells. Arrowheads, intracellular compartment positive for DPP IV, LAMP1 and Cathepsin D. Scale bar: 10 μm.
data suggest that apical proteins are re-routed to the degradation pathway upon Drebrin E depletion. To confirm our observations that the occurrence of this large intracellular compartment is linked to Drebrin E depletion, we quantified the number of these compartments containing both LAMP1 and an apical marker (DPPIV, SI or IAP) both in CT and Drebrin E KD cells. We found that the percentage of cells containing intracellular compartments positive for both an apical membrane protein and LAMP1 went from 1% for CT cells to up to 17% for Drebrin E KD cells (not shown, see Material and Methods). Rescue experiments with mouse Drebrin E caused a strong disorganization of the F-actin cytoskeleton leading to aberrant cell shape and to rapid cell death (20). We thus could not use this transient over-expression of mouse Drebrin E to rescue the accumulation of apical membrane proteins in the KD cells. Altogether, these data demonstrate an enrichment of apical membrane proteins in a degradation compartment upon Drebrin E depletion.

Apical membrane proteins accumulate in enlarged lysosomes after endocytosis

Our hypothesis to explain the accumulation of apical proteins in the degradation pathway after Drebrin E depletion was that it reflected a perturbation of an apical route. This could also explain the decrease of apical labeling for apical membrane proteins (Fig. 1B). We thus tested whether Drebrin E depletion affected apical membrane protein levels by measuring the total amount of DPPIV and SI proteins in CT and Drebrin E KD cells at D5 after transfection. We found that the levels of DPPIV and SI were indeed reduced, to 35 and to 20%, respectively, in Drebrin E KD compared with CT cells (Fig. 3A and B). Conversely, Ag525, a basolateral marker, was not significantly affected in the same conditions (Fig. 3A and B).

We then questioned whether the observed drop in steady state levels of apical proteins originate from a decrease in apical proteins biosynthesis or an increase in apical protein degradation, or both. To test whether biosynthesis is affected, we measured the total amount of apical protein synthesized after a radioactive pulse followed by an immunoprecipitation of the apical proteins. After the radioactive pulse, we observed a strong decrease in biosynthesis of total amount of both SI and DPPIV in Drebrin E KD cells, whereas Ag525 was less affected (Fig. 3D). These data suggest that the reduction of apical protein staining and their expression levels in Drebrin E KD cells could be explained mainly by a strong decrease in their biosynthesis. Then, we asked whether this decrease in biosynthesis could correlate with the decrease in DPPIV or SI mRNA production that was measured by quantitative RT-PCR in CT or Drebrin E KD Caco2 cells (Fig. 3C). Surprisingly, SI mRNA levels were strongly affected by Drebrin E depletion but not DPPIV or E-cadherin mRNA levels, indicating that each mRNA or protein has a specific regulation.

Because apical proteins appear to undergo inappropriate trafficking in Drebrin E KD cells, we asked whether this mistargeting occurred after their biosynthesis or during their apical recycling. To examine the first hypothesis, we used a combination of radioactive pulse-chase and cell surface biotinylation (21). We quantified the respective amounts of SI and DPPIV transported to the apical membrane (by a direct or an indirect pathway, respectively) and Ag525 to the basolateral membrane after biosynthesis [see Material and methods and (22)]. No differences were observed between CT and Drebrin E KD cells for the delivery of each of these proteins after 10 h of chase, suggesting that the post-Golgi targeting of apical and basolateral proteins was not affected by Drebrin E depletion (Fig. 3D).

Because apical protein mistargeting does not occur during their biosynthetic transport to their target membrane, we assumed that the apical protein accumulation in large lysosomes could originate from apical endocytosis in the Drebrin E KD cells. To investigate this issue, we developed an endocytic assay for DPPIV in Caco2 cells. Living CT or Drebrin E KD cells were incubated at 4°C with antibodies against DPPIV from the apical surface. Then, after cold washes, cells were either warmed for 30 min at 25°C to prevent, or at 37°C, to allow for endocytosis. Subsequently, cells were fixed and permeabilized and then labeled with secondary antibodies to reveal the internalized pool of DPPIV (Fig. 4A). At non-permissive temperature (25°C), no accumulation of DPPIV-specific antibodies in LAMP1-positive organelles in both CT and KD cells was detected (Fig. 4A). When cells were allowed to internalize the antibodies at 37°C, we found an enrichment of DPPIV antibodies in enlarged lysosomal structures exclusively in Drebrin E KD cells (Fig. 4A). These data were confirmed in an experiment in which living CT or Drebrin E KD cells were incubated overnight at 37°C with antibodies against DPPIV present in the apical medium and then processed as described earlier. Again, we found the accumulation of DPPIV signal in LAMP1 structures (not shown). In addition, these intracellular organelles were different from the apical invaginations we could also detect deep into the monolayer both in CT and Drebrin E KD cells (see Fig. 5A, arrows in CT cells and Supplementary Material, Fig. S2C for a cartoon). We thus demonstrated that the pool of apical proteins present in these enlarged lysosomes originated from apical endocytosis. There was, however, no significant change in apical endocytosis as measured by the amount of a rhodamine-labeled dextran that was taken-up from the apical side either in CT or Drebrin E KD Caco2 cells (Fig. 4C). In addition, some of this apically endocytosed fluorescent dextran was accumulated in the large lysosomal structures in Drebrin E KD cells, confirming that these compartments are accessible from the apical domain (Fig. 4B). Altogether, these results suggest that in Drebrin E KD cells, two mechanisms concur to reduce the level of apical proteins. The first one is the decrease of their biosynthesis and the second is based on their accumulation in a lysosomal compartment after apical endocytosis.

Rab8a depletion mimics Drebrin E depletion with the enrichment of apical proteins in enlarged lysosomes

All these data suggested that there might be a defect in the apical recycling pathway in Drebrin E KD cells. Several proteins are involved in the recycling pathway, and among them, Rab8a plays an important role in apical membrane formation (12). Furthermore, it has been previously shown in a Rab8a cKO mouse that apical membrane proteins are accumulated in enlarged lysosomes in enterocytes. The Rab8a cKO mice enterocytes also showed shorter microvilli (14), a phenotype reminiscent of the one we observed upon Drebrin E depletion (20). To further demonstrate a possible functional interaction between Rab8a and Drebrin E in apical recycling, we transiently depleted Rab8a from Caco2 cells for 5 days (as for Drebrin E KD), which resulted in the formation of an enlarged compartment positive for both
DPPIV and LAMP1 (Fig. 5A), similar to that observed in Drebrin E knock-down Caco2 cells. As for Drebrin E KD cells, no accumulation of F-actin was detected in these large intracellular vesicles upon Rab8a depletion, whereas rare microvillar inclusions were described in vivo in the Rab8a cKO mouse (14). The brush border was highly affected with sparse and shorter microvilli very similar to the Drebrin E KD cell phenotype (Fig. 5B). To ensure that the formation of an enlarged degradation compartment was not an in vitro artifact, we compared this phenotype with the one observed on intestinal sections from Rab8a cKO mouse. As expected, in Rab8a cKO mouse enterocytes, IAP was enriched in a subapical enlarged compartment positive for LAMP2, a marker of the degradation pathway (Fig. 6). No actin or Villin was detected in these subapical structures, confirming that they are related to the degradation compartments and not to microvillar inclusions. No such intracellular accumulation of apical membrane proteins was observed in wild-type (CT) littermates (Fig. 6). These results

Figure 3. Apical membrane protein expression is decreased, but polarized surface targeting is not affected in Drebrin E knock-down Caco2 cells. (A) Immunoblot analysis of apical (SI and DPPIV) and basolateral (Ag525) membrane protein expression levels in CT and Drebrin E knock-down (Dreb KD) cells. α-Tubulin is used as a loading control. Molecular weights are indicated on the left (kD). (B) Apical and basolateral membrane protein expression levels in CT (dark gray bars) and in Drebrin E knock-down cells (Dreb KD—light gray bars). Error bars represent the SD. DPPIV (N = 4); SI (N = 9); Ag525 (N = 3). (C) Quantitative RT-PCR of mRNA in CT and Drebrin E knock-down (Dreb KD) cells. E-cadherin (a basolateral marker), DPPIV and SI (two apical markers) mRNA levels in Drebrin E KD cells were expressed as a relative amount of the corresponding Cts. (D) Left column: Ag525, DPPIV and SI protein biosynthesis in CT (dark gray bars) and Drebrin KD (light gray bars) cells, N = 3 independent experiments. Newly synthesized proteins were metabolically labeled with [35S]-cysteine and [35S]-methionine and then immunoprecipitated with antibodies against Ag525, DPPIV and SI. Right column: cell surface targeting of Ag525, DPPIV and SI after metabolic labeling and biotinylation of apical and basolateral membrane in CT (dark gray bars) and Drebrin E KD (light gray bars) cells. Ag525, DPPIV and SI were immunoprecipitated with their respective antibodies, and their expression level on the apical and basolateral membranes was quantified, N = 4 different filters. Error bars represent the SD.
strongly suggest that the compromise of Rab8a either in cells or in mice results in phenotypes that are highly reminiscent of Drebrin E depletion, namely with the inappropriate accumulation of apical proteins in large lysosomes.

To further understand a possible functional interaction between Drebrin E and Rab8a in intestinal cells, we stably over-expressed GFP::Rab8a in Caco2 cells and then performed a transient depletion of Drebrin E as described earlier. Drebrin E KD induced the formation of large apically enriched lysosomes both in GFP::Rab8a-positive (regardless of the level of GFP::Rab8a overexpression) and GFP::Rab8a-negative cells, indicating that overexpression of Rab8a protein cannot compensate for the loss of Drebrin E in Caco2 cells (Supplementary Material, Fig. S3).

As Rab8a and Drebrin E depletion phenotypes in Caco2 cells were very similar, we examined the expression and localization of Drebrin E in the Rab8a eKO mouse model and in the Rab8a KD Caco2 cells. In Rab8a eKO mouse intestine, there was a decrease of subapical Drebrin E staining when compared with its CT counterpart (Fig. 6, upper panel). This change in

Figure 4. DPPIV is enriched in late endosomal/lysosomal compartment after endocytosis from the apical membrane. (A) DPPIV (red) and LAMP1 (green) localization at non-permissive (−) or permissive temperature (+) for endocytosis in CT (upper panels) or Drebrin E knock-down (Dreb KD, bottom panels) cells. Arrows, apical membrane invaginations; arrowheads, late endosomal/lysosomal compartment. Nuclei are labeled with DAPI (blue). Scale bar: 10 μm. (B) Internalization of rhodamine–dextran (TRITC-Dext) in CT (upper panels) or Drebrin E knock-down (Dreb KD, bottom panels) cells. Arrowheads, co-localization of internalized rhodamine–dextran (TRITC-Dext) with lysosomal compartments. Scale bar: 10 μm. (C) Quantification of apically internalized rhodamine–dextran in CT (dark gray bars) or Drebrin E knock-down (Dreb KD—light gray bars) cells.

Figure 6.
Drebrin E labeling could be due either to dispersion into the enterocytes cytoplasm or to a decrease in Drebrin E levels. To test these hypotheses, we quantified the level of Drebrin E expression by immunoblot in Caco2 cells depleted for Rab8a compared with CT cells (Fig.5C). A slight but significant decrease in Drebrin E expression level was observed (18% less when compared with CTs) and the reverse was also true (22% less when compared with CTs), indicating that the levels of each of the two proteins might be, at least in part, regulated by the other. More interestingly, using confocal microscopy, we found a change in apical Drebrin E distribution upon Rab8a depletion with a decrease in subapical staining (Fig. 5A), indicating that Rab8a might regulate Drebrin E localization and/or organization in intestinal cells either directly or indirectly.

**DISCUSSION**

Drebrin proteins are mainly known for their function in neuronal morphogenesis. However, these proteins are widely expressed in non-neuronal tissues where their function is still unclear (19). We have previously shown that Drebrin E regulates epithelial cell morphogenesis (20). In this study, we highlight a new function of Drebrin E as a key protein involved in the regulation of apical trafficking in Caco2 cells.

We found that upon Drebrin E depletion, Caco2 cells accumulate apical membrane proteins in a large intracellular compartment related to lysosomes. These intracellular compartments do not contain microvilli and are not surrounded by a terminal web as we demonstrated here by the absence of F-actin or...
phospho-myosin II staining around them in Drebrin E KD cells. However, these organelles contained several apical markers, and in parallel, the brush border on the apical membrane was dramatically affected. Thus, these intracellular organelles positive for apical markers were different from the intercellular apical membranes, called vacuolar apical compartments (VAC), described earlier in HT-29 cells (23) and in MDCK cells (24). Moreover, VAC formation resulted from the disruption of the microtubule network (25). In Drebrin E KD cells, the microtubule organization is only modestly affected, although EB3, a plus-end microtubule binding protein, does not accumulate at the terminal web owing to Drebrin E depletion (20). We also demonstrate here that the accumulation of apical membrane proteins in lysosomes results from their endocytosis and we know that VACs originate from biosynthesis and not from endocytosis of the apical plasma membrane (24). There are several observations that corroborate this lysosomal identity for this compartment including the presence of specific markers of the degradation pathway such as LAMP1, CD63 and Cathepsin D [for review see (26)]. The heterogeneous content is consistent with this being a lysosomal compartment.

The fact that we could observe an important accumulation of apical membrane proteins in lysosomes indicates that the degradation function of these structures was somehow impaired. This could be due to the massive delivery of apical membrane to pre-lysosomal compartments, thus overloading them and leading to a reduction in turnover efficiency. This impairment of lysosomal degradation might be due to either a misrouting in the delivery of lysosomal enzymes to the lysosomal compartment to fulfill their function or to defects in their folding or their post-translational modification. We currently do not know whether and how Drebrin E depletion could affect the delivery or the maturation of lysosomal enzymes, and we did not detect any ectopic labeling for lysosomal markers, indicating that misrouting is not a likely hypothesis. Further work will help to elucidate this function in greater detail.

Nevertheless, we demonstrated here that apical membrane proteins are normally delivered to the plasma membrane after biosynthesis and are then endocytosed from the apical

Figure 6. Apical membrane proteins relocalize to late endosomes/lysosomes, and Drebrin E localization is affected in Rab8a conditional knock-out (KO) mouse intestine. Upper panels, Drebrin E (green) and actin (red) localization is weaker at the terminal web and laterally and late endosome/lysosome compartment (LAMP2-positive compartment—cyan) is enlarged in Rab8a KO intestine compared with control intestine (CT). Lower panels: apical membrane proteins (IAP—red) co-localize with the late endosomal/lysosomal compartment in Rab8a KO but not in control intestine. Apical cytoskeleton (Villin—green) does not co-localize to the late endosomal/lysosomal compartment. LP, lamina propria; arrowheads, late endosomal/lysosomal compartment; bracket, microvilli. Nuclei are stained with DAPI (blue). Scale bar: 10 μm.
membrane only to accumulate in the lysosomal compartment in Drebrin E KD cells. As apical membrane proteins are not detected intracellularly in CT cells, we hypothesized that upon Drebrin E depletion apical proteins that are endocytosed are not properly recycled to the apical surface because we did not observe an increase in apical endocytosis using a fluid phase marker. Rab11 a/b has been shown to regulate apical protein recycling together with Rab25 (27). There are, however, no report of accumulation of apical membrane proteins in such large intracellular lysosomal compartments upon Rab11 a/b depletion in intestinal cells, indicating that they were either not investigated or not found to date (28). Rab8a is another protein of the Rab GTPase family involved in the recycling pathway [for review see (29)], and its deletion in a mouse cKO model was associated or not found to date (28). Rab8a is another protein of the Rab GTPase family involved in the recycling pathway [for review see (29)], and its deletion in a mouse cKO model was associated or not found to date (28).

We here demonstrate a similar phenotype in intestinal cells depleted for Drebrin E (this study). The common phenotype between Drebrin E and Rab8a depletion in Caco2 cells indicates that both proteins act in the apical recycling pathway by a yet-unknown mechanism. Interestingly, while overexpression of GFP::Rab8a did not rescue Drebrin E depletion in Caco2 cells, Rab8a depletion induced a loss of Drebrin E accumulation in the subapical domain of intestinal cells both in vivo and in vitro. These data confirm that a functional link (that might be indirect) exists between Rab8a and Drebrin E. The exogenously expressed GFP::Rab8a did not bind Drebrin E in immunoprecipitations; therefore, it is unlikely that the two proteins associate under normal cellular conditions (data not shown). If Drebrin E and Rab8a act in the same pathway, it could be through their role in their cognate functions in affecting subapical acto-myosin organization. Indeed, we have previously shown that upon Drebrin E depletion, there was a dramatic reduction of the terminal web, a subapical acto-myosin dense meshwork, in Caco2 cells (20). Rab8a cKO in intestine on the other hand clearly disrupts apical actin organization [(14), this work]. Taken together, these observations suggest that Drebrin E and Rab8a depletion affect the subapical actin cytoskeleton that is essential for the appropriate regulation of apical trafficking (30).

MyoVb is a direct effector of both Rab8a and Rab11a to CT epithelial polarization in MDCK cells (10) and is a known regulator of apical recycling pathway [for review see (7)]. Furthermore, MyoVb depletion in Caco-2 cells exhibited a phenotype (9) with common features with that described for Drebrin E and Rab8a in our study. One key feature that so far distinguish the MYO VB or Rab8a KO in vivo phenotypes from the other key features is the deletion of the Drebrin A isoform, likely due to a compensatory up-regulation of Drebrin E (35). Further in vivo and genetic investigations will be needed to ascertain the connection between Drebrin E and the MyoVb/Rab8a/Rab11a complex.

MATERIAL AND METHODS

Cell culture

TC7 cells, a Caco-2 clone was grown as previously described (36). Resuspended cells were transfected with Amaxa device (B-024 program for siRNA and T-020 for plasmids, Amaya Biosystems, Germany) and the following mix: 100 pmol of siRNA, Nucleofactor buffer T and 2 × 10^5 of freshly trypsinized cells in line with the manufacturer’s instructions. Knock-down of Drebrin E expression was monitored by western blot and immunofluorescence at D3, D4 or D5 after transient depletion. Caco2 cells were transfected with Amaya device and a plasmid encoding human GFP::Rab8a with OptiMEM ( Gibco) 2 mg/ml for 1 week and then 1 mg/ml and maintained with 0.2 mg/ml. For Rab8a transient depletion, Caco2 cells in suspension were transfected with Lipofectamine 2000 (Invitrogen) and the following mix: OptiMEM (Gibco), 100 pmol of siRNA, 10 μl of Lipofectamine 2000 and 5 × 10^5 of freshly tryptsinized cells in line with the manufacturer’s instructions. Cells were seeded on Transwell filters (24 mm in diameter, Corning, NY, USA) for immunofluorescence assays.

siRNA and constructs, RT-PCR

The following siRNA were used: human Drebrin E and CT siRNAs were previously described (20), a pool for human Rab8a siRNAs, only sense sequences are indicated: (1) 5′-GAA-CUGGAUUCGCAAACAUU-3′, (2) 5′-GAACAGUGUGA-GUGAUU3′, (3) 5′-GAUUAACUGCAGAU-3′ and (4) 5′-CAGGAACGGUUUCGCCAGCA-3′, (ON-Target Plus Smart pool, Dharmacon). The plasmid pEGFPC-Rab8a (wild type) was from A. Echard (Institut Pasteur, Paris, France). Total RNA was extracted from Caco2 cells according to the

Human Molecular Genetics, 2014, Vol. 23, No. 11
instructions for the use of the PARIS kit (Applied Biosystems). Each RNA sample was reverse-transcribed into the cDNA using the High Capacity RNA-to-cDNA master mix (Applied Biosystems). Taqman Gene Expression Assays were used to detect endogeneous s18 ribosomal protein, E-cadherin, DPPIV and SI. The PCR was performed on the 7500 fast real-time PCR system.

Antibodies

The following primary antibodies were used: guinea pig polyclonal anti-Drebrin was used for immunofluorescence assays (Progen Biotechnik), mouse monoclonal anti-Drebrin was used for western blotting (Medical and Biological Laboratories), chicken polyclonal anti-GFP (Aves Laboratories) was used for immunofluorescence and mouse monoclonal anti-GFP (clones 7.1 and 13.1) (Roche), rat monoclonal anti-DPPIV (clone 4H3) produced by D. Massey-Harroche were used for immunofluorescence and western blotting. Mouse monoclonal anti-Sucrase-Isoamaltase (clones 3.73 and 4.34) (a gift from A. Quaroni, Ithaca, NY, USA) was used for immunofluorescence and western blotting, and rabbit polyclonal anti-Intestinal Alkaline Phosphatase (IAP, a gift from G. Rougon, IBDM, Marseille, France) was used for immunofluorescence and western blotting. Sheep polyclonal anti-TGN46 (AbD Serotec), mouse monoclonal anti-EEA1 (BD Transduction Laboratories), mouse monoclonal anti-Rab11a (clone 7C10, Santa Cruz biotech.), goat anti-Cathepsin D (Santa Cruz biotech.) and rabbit anti-phospho-myosin II (Cell signaling tech.) antibodies were used for immunofluorescence and western blotting. Mouse monoclonal anti-LAMP1 (clone H4A3, Biolegend) was used for immunofluorescence and western blotting, and rat monoclonal anti-LAMP2 (clone ABL-93, Developmental studies Hybridoma Bank) for immunofluorescence, goat polyclonal anti-Rab8a (AbD Serotec) for western blotting. Mouse monoclonal anti-CD63 (a gift from J. Salamero, Curie Institute, Paris, France) and mouse monoclonal anti-Villin (clone 5.1) (a gift from D. Louvard, Curie Institute, Paris, France) were used for immunofluorescence. Antibody anti-Ag525 was previously described (37). Mouse monoclonal anti-α-Tubulin (clone B-5-1-2, Sigma–Aldrich) and β-actin with rabbit monoclonal (clone 13E5, Ozyme). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma–Aldrich) was used to label the nuclei. The corresponding secondary antibodies coupled to horse-radish peroxidase (Jackson ImmunoResearch) were used for western blotting and those conjugated to FITC, Cyanine-3, Cyanine-5, Alexa 488 or Alexa 647 were used for immunofluorescence.

Western blotting

Caco2 cells were extracted using the following lysis buffer: Tris 50 mM (pH 8), NaCl 150 mM, EDTA 2 mM, NP40 0.5%, and Complete Mini Protease Inhibitors (Roche) and analyzed by the previously described western blot procedure (38). Bands were revealed with LumiLight kit (Roche Diagnostic, Healthcare) and quantified with ImageJ software program (NCBI).

Pulse-chase and immunoprecipitation procedures

Transfected Caco2 cells were grown on Transwell filters for 5 days before 30 min of incubation in DMEM without cysteine/methionine, pulsed for 30 min in the same medium containing 37 MBq of [35S] methionine and [35S] cysteine as described (39). Cells were washed once with DMEM, then chased for 0 or 10 h in DMEM containing 10× cysteine/methionine and stored at 4°C in NaCO3H-free DMEM/20 mM HEPES/0.2% bovine serum albumin (BSA) before biotinylation. Biotinylation of apical or basal surface of cell monolayer on Transwells and the following double-immuno and streptavidin precipitations to isolate the surface-labeled newly synthesized proteins were performed as previously indicated (39). Protein biosynthesis at T = 0 h (N = 3 independent experiments ± SD) and cell surface targeting at T = 10 h were quantified with ImageQuant software (N = 4 independent filters ± SD).

Cell immunofluorescence

Transfected Caco2 cells were seeded on Transwell filters and maintained for 3, 4 or 5 days in vitro. Cells were washed with PBS, fixed with 3% paraformaldehyde for 15 min and permeabilized for 10 min with 0.5% Triton X-100. Cells were blocked with 0.4% gelatin and 0.025% saponin for 1 h before being incubated with primary antibodies overnight at 4°C. After being incubated with the appropriated fluorescence-conjugated secondary antibodies, cells were washed and mounted in DABCO/Mowiol anti-fading reagent. Images were acquired with LSM 510 Meta or LSM 780 microscope using a 40× Plan-Apochromat (1.2 NA water) objective (Zeiss, Le Pecq, France).

Rab8 conditional knock-out mouse intestine immunofluorescence

Rab8a conditional knock-out mice have been previously described (14). Intestinal paraffin-embedded sections of 8-week-old mice were treated for 15 min with antigen unmasking solution (Vector) prior to immunofluorescence using standard protocols (14). Images were acquired using confocal microscopy, as for Caco2 cells.

Electron microscopy

TEM: Transfected Caco2 cells were seeded on Transwell filters and maintained for 5 days in vitro. Cells were treated for TEM as previously described (40). Immunogold: Transfected Caco2 cells were seeded on Transwell filters and maintained for 5 days in vitro. Then, cells were treated as previously described (40). Sections were incubated overnight at 4°C with previously described primary antibodies (anti-LAMP1 and anti-DPPIV) in 0.05 m Tris-buffered solution (pH 7.6) with 0.1% BSA and 0.05% Tween 20. After washing, the sections were incubated with anti-chicken or anti-rat antibodies coupled to 6- or 15-nm gold particles (Aurion), respectively, in the same buffer. Samples were processed as described (41) and observed on a Zeiss 912 electron microscope (Zeiss).
Endocytic assay
Transfected Caco2 cells were seeded on Transwell filters and maintained for 5 days in vitro. For the dynamic endocytic assay, apical Caco2 medium was replaced by a hybridoma supernatant containing an anti-DPPIV antibody (clone 4H3) 30 min at 4°C. The hybridoma supernatant was replaced by the apical Caco2 medium, and cells were placed 30 min at non-permissive temperature (25°C) or at permissive (37°C) temperature for anti-DPPIV endocytosis. Ten kilodaltons of Rhodamine-labeled Dextran (Sigma–Aldrich) was used with a 45-min internalization time at 37°C [42]. Alternatively, a cumulative endocytic assay used transfected Caco2 cells maintained for 4 days in vitro, and then, the apical Caco2 cell medium was replaced by the previously described hybridoma supernatant and placed 16 h at permissive temperature (37°C) for anti-DPPIV cumulative endocytosis. For both endocytic assays, cells were washed three times with PBS and then processed for immunofluorescence as previously described. The previously described primary antibodies used were mouse anti-LAMP1 and guinea pig anti-Drebrin, and the fluorescence-conjugated secondary antibodies used were anti-mouse, anti-guinea pig and anti-rat. Images were quantified using the Image J software.

Statistical analysis
Percentage of endosomes/lysosomes was established by the quantification of compartments positive for apical marker and LAMP1 in CT and Drebrin-depleted Caco2 cells. Three independent experiments were quantified. A Student’s t-test was performed for statistical analysis (P < 0.05). Number of late endosomes/lysosomes positive for apical markers (DPPIV and IAP) in CT (N = 1966 cells) and Drebrin knock-down (N = 1066 cells) cells.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We thank the Le Bivic Lab members, the IBDM imaging facility for help with imaging experiments and Jean-Paul Chauvin (IBDM) for the electron microscopy. We also thank Richard Roy (McGill University, Canada) for his critical reading of the manuscript.

Conflicts of interest statement. None declared.

FUNDING
We acknowledge France-BioImaging infrastructure supported by the Agence Nationale de la Recherche (ANR-10-INSB-04-01, call ‘Grand Empreun’). This research was supported by the Centre National de la Recherche Scientifique (UMR7288) and Aix-Marseille University. The Le Bivic group is an ‘Equipe labellisée 2008 de la Ligue Nationale contre le Cancer’. B.V. was the recipient of a fellowship from Grant agreement number (HEALTH-F2-2008-200234) and from the French ‘Association pour la recherche sur le cancer’ ARC. This project was supported by Coordination Theme 1 (Health of the European Community’s FP7, Grant agreement number (HEALTH-F2-2008-200234 and ANR n°BLAN07-2-186738).

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