Pathogenic mechanisms in microvillus inclusion disease
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Summary

The microvilli brush border in polarized epithelial cells is the most pronounced structural aspect of the apical plasma membrane and an important parameter regulating cell homeostasis. The development of the apical brush border is the pinnacle of morphological adaptation of cells in biological niches to suit their functions. Microvilli cover the apical surface of the intestinal epithelial cells and contribute to absorption (or secretion in a sub-type of epithelial cell) of water and nutrients. As the intestinal epithelial cells migrate from the crypts to the tips of the villi, gradually reaching terminal differentiation, microvilli too assemble, mature, elongate, and develop into a characteristic tightly packed organized organelle. (chapter 1). Disorganized microvilli accompany many pathologies of the gut underscoring their importance in gut homeostasis. The function of microvilli reaches beyond that of increasing absorptive surface area and include functions related to, among others, innate immunity, cell migration, and tumor suppression. The development of microvilli is still ill-understood and involves several intracellular signaling pathways and cytoskeletal proteins. How the endomembrane system contributes to apical microvilli development is not known. In this thesis we have studied the pathogenesis of an intestinal disorder, microvillus inclusion disease (MVID; OMIM 251850), that is characterized by mutations in the endomembrane system-regulating protein myosin Vb, and apical plasma membrane defects including microvillus brush border atrophy. Neonates suffer from severe diarrhoea and mal-absorption, and their generally poor prognosis depends on life-long total parenteral nutrition and intestinal transplantation. MVID is associated with mutations in the MYO5B gene, which codes for the actin motor protein myosin Vb. All the mutations known yet (published and some unpublished) in the various regions of the MYO5B gene and their possible biological implications have been reviewed in chapter 2. This has resulted in the construction and launch of an MVID patient registry and database, www.mvid-central.org, which tracks and maintains updated knowledge on MVID patients, their MYO5B mutations, and clinical course of their disease. Bringing such knowledge together is important to study the structure-function relationship of myosin Vb and to perform genotype-phenotype correlation studies.

An important protein implicated in the organization of the actin-rich brush border is ezrin. The phosphorylation and thereby the activation of ezrin at Thr567 that allows the protein to unfold and link the actin filaments to the apical membrane and is a pivotal step in the machinery controlling microvilli organization. In chapter 3, we demonstrated that kinases that phosphorylate ezrin (MST4, aPKCi, and its activating partner PDK1) are
subapically localized. These kinases co-distribute in vivo and in vitro with rab11a positive apical recycling endosomes. ShRNA-mediated myosin Vb downregulation in enterocytes redistributes these rab11a-recycling endosomes and the kinases away from the apical region and thereby inhibits ezrin phosphorylation at the apical domain. Importantly, in enterocytes of MVID patients, rab11a and the kinases are similarly mislocalized to supra-nuclear region, and ezrin phosphorylation is inhibited. We thus defined a new mode of brush border control by recycling endosomes arguing for a novel role for these endosomes as dynamic signaling platforms (Figure 1).

We found in chapter 4 that another GTPase Rac1, studied for its role in cell polarity, migration, and actin reorganisation, is present in vivo and in vitro at the apical membrane within the microvilli. This apical localization is dependent on myosin Vb expression. Importantly, Rac1 is absent from the apical domain in MVID enterocytes and enriched in supra-nuclear punctae that are also positive for rab11a. This implies that Rac1 may be involved in the pathogenesis of MVID. We tested this hypothesis by down-regulating Rac1 in enterocytes which led to reduced ezrin phosphorylation and disorganized microvilli on the apical surface. This effect is independent of its role in cell-cell adhesion. Regulation of ezrin activity did not depend on the GTPase cycling of Rac1 or on the localization of recycling endosomes. Rac1 controls the enrichment and subapical localization of the ezrin-phosphorylating kinase MST4. An intestine specific Rac1 knockout mouse also displays a similar phenotype pointing towards a novel role of Rac1 as a scaffolding protein which maintains the apical enrichment of components needed for brush border maintenance (Figure 2).

Familial histophagocytic lymphohistiosis type 5 (FHL5) is an immune disorder caused by mutations in fusion machinery protein munc18-2. Some patients show signs of a diarrheal disorder with cellular phenotypes comparable to MVID. Therefore, in chapter 5 we investigated the functional relationship between MYO5B and the apical fusion machinery. We found that the apical targeting of the key brush border membrane fusion protein syntaxin-3, a t-SNARE that is important for general protein delivery to the brush border, is dependent on myosin Vb expression. Syntaxin-3 also contributes to MVID pathogenesis as its shRNA-mediated
downregulation affects brush border organization and ezrin phosphorylation. This functional link between syntaxin-3 and ezrin is reinforced by studying FHL5 intestine wherein crypts, but not villi, show intra-cellular mis-localized syntaxin-3 and inactive apical ezrin. Such cytoplasmic retention of the apical membrane fusion machinery explains the global defect in apical membrane organization in MVID enterocytes. Thus, studying apparently unrelated congenital disorders led us towards identifying a unifying molecular pathway causing a subset of diarrheal disorders (Figure 3).

Diarrhea in MVID patients is intractable and of secretory origin. We tested in chapter 6 whether the epithelial lining was functional as a barrier to fluid. Analyses of the subcellular distribution of key tight junction proteins revealed that myosin Vb depletion affects the intra-cellular localization and trafficking of tight junction sealing protein, claudin-1, but not of any other examined tight junction / adhesion junction proteins in intestinal epithelial cells. This mis-localization and retention of claudin-1 does not increase the inter-cellular permeability of macromolecules. The correct localization of other junctional proteins helps maintain the apico-basal polarity and tissue-barrier integrity of the intestinal epithelia. The intractable secretory diarrhea is not caused by epithelial barrier defect and may have an alternative origin.

In conclusion, we have elucidated novel mechanisms that contribute to brush border organization, implicated the endomembrane system as an important regulatory factor, and gained insights into the mechanism underlying the pathogenesis of microvillus inclusion disease.

**Future perspectives**

Prior to the knowledge of the role played by apical recycling machinery described in this thesis, there was limited knowledge on membrane trafficking pathways that could uncouple the brush border development from the general polarity program at the molecular level in epithelial cells. Our findings are supported by localization studies across intestinal epithelial cell culture models and in vivo models of intestinal tissues. The study of the apical plasma membrane recycling machinery and its co-distribution with important kinases and fusion machinery in the context of microvillus inclusion disease strengthen our in vitro observations.

The next step would be to identify factors (specific lipids or proteins) that are necessary for the recruitment of the kinases to the recycling endosomes. Through our studies, there has emerged a novel role for recycling endosomes in the form of molecular platforms which harbour signaling proteins and regulate microvilli dynamics. Using advanced techniques like super-resolution microscopy and proteomics, individual molecular components could be identified and isolated events be visualized with regard to the assembly of supra-molecular complexes on apical recycling endosomes. Moreover, the role of endo-membranes in trafficking and oligomerization of cytoskeletal / membrane-linker proteins such as actin / ezrin in the subapical niche is an open question yet to be answered. Our findings on the recycling, endosome-dependent, apical trafficking of Rac1 and its role in microvilli organization by selectively regulating the subapical positioning of
MST4, underscores the importance of identifying scaffold proteins at the apical recycling endosomes. Moreover, interaction studies between Rac1 and components such as rab11a and MST4 (or its recruiter TNIK) could provide mechanistic insight with respect to their recruitment to recycling endosomes. The observation that Rac1 regulates ezrin phosphorylation in the brush border in both GTP- or GDP-bound forms, demands further attention as, not often have roles been

Our investigations into MVID pathogenesis have raised new questions on the regulated trafficking of cytosolic and membrane-bound apical fusion machinery. The discovery, that rab11a (apical endosome regulator) and syntaxin-3 (regulator of fusion at the brush border) are co-mislocalized in MVID enterocytes, raises the question as to whether the apical targeting and functioning of both proteins is inter-dependent. Visualizing apical-directed cargo by live imaging in myosin Vb depleted cells might help answer a critical gap in our understanding of MVID: Is the trafficking problem a defect of biosynthetic delivery (mediated by syntaxin-3) or of membrane recycling (mediated by rab11a) or of both? Moreover, recent knowledge that degradative pathways connect to the recycling endosomes compels one to think about role of autophagy in the eventual degradation of the mislocalized brush border membrane proteins in MVID enterocytes.

The lack of suitable models has hampered our understanding of cause-effect relationship between mutations and the phenotype. The development of myosin Vb knockout mouse might help us unravel pathogenic mechanisms causing gross morphological changes in the MVID intestine, but also help us study the cause of fatal secretory diarrhea seen in MVID patients. We identified that junctional protein claudin-1, even if mis-positioned after myosin vb depletion, does not affect epithelial barrier function. But several other proteins such as claudin-2, -15, critical for regulating paracellular fluid flow might be de-regulated and contribute to this pathogenesis. Use of newer models such as intestinal organoids could also give us a handle to examine chronological events in brush border assembly along the life-time of a cell helping visualize intestinal morphogenesis, especially, if they were made from cells of MVID patients.

In conclusion, polarity and microvilli is an emerging field in cell biology. An improved understanding of regulated endosome networks, physiologically relevant model systems, protein interactomes, and the further integration and development of microscopic techniques are the way to identify key molecules that rescue the brush border atrophy in life-threatening disorders such as microvillus inclusion disease.