Chapter 1

INTRODUCTION TO BRUSH BORDER DEVELOPMENT AT THE APICAL MEMBRANE

&

SCOPE OF THE THESIS

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Abstract

Polarized epithelial cells have their surfaces differentiated into apical and basolateral membrane domains separated by tight junctions. Several mechanisms work in concert to ensure that this apico-basal polarity is established and maintained, one of the most important factors being intracellular trafficking of cargo. The apical membrane is constantly restructured and maintained by regulated endocytosis and recycling, sorting of the apical directed cargo, selective retention of apical proteins, and displacement from the basolateral membrane. These polarity cues also play a role in development of the structural aspect of the apical membrane, called microvilli. The composition of the microvilli has been well studied but the elucidation of their formation and dynamic nature remains elusive. Cytoskeletal proteins and their associated factors contribute to providing rigidity and structure to the apical domain but whether they contribute in initiating this unique morphology is yet uncertain. Intra-cellular signaling cascades of cytosolic kinases and adaptor proteins also contribute to the dynamic nature of the brush border. Some of these signaling pathways are important for both, the establishment of apico-basal polarity and the formation of microvilli. Yet microvilli formation is not an inevitable outcome of apico-basal polarity in epithelia and can be de-coupled at the molecular level. Importantly, the presence of regulators of vesicular trafficking in microvilli-related signaling cascades makes one wonder whether endo-membranes’ flow have a bigger role than previously thought of in microvilli assembly. This chapter reviews the recent advances in the molecular cross-talk of microvilli-related signaling cascades and touches on novel roles for microvilli, apart from the commonly known role in expanding the apical surface area.

Introduction to general epithelium:

Epithelial cells line the interface between the organism and the exterior world in the form of monolayers covering the vast areas of the digestive, respiratory, urinary and reproductive tracts. Epithelia are important in maintaining the homeostasis by vectorial transport of nutrients, ions, and waste and, therefore, are intrinsically asymmetrical or polarized (Rodriguez-Boulan, 2008). Cell polarity can be defined as the anisotropic distribution of cellular components to perform distinct functions. The epithelial cell membrane is divided into the apical domain which faces the “outside” luminal space and basolateral domain which faces “inside” tissue contacting the basement membrane, extracellular matrix, blood capillaries, as well as the basolateral domain of the neighbouring cell. The two domains are physically separated by tight junctions (TJs), a critical component in the establishment of epithelial cell polarity. TJs are typically situated at the apical-most region of the lateral membrane and serve as an intercellular barrier that limits paracellular permeability (Keith Mostov et al, 2008) and intra-membranous fence that limits lateral diffusion of apical and basolateral membrane proteins (Giepmans and van IJzendoorn, 2009). The apical and basolateral membranes face different environments and have distinct functions that are accompanied by dissimilar lipid and protein compositions. With the polarization of the cell, there is also an asymmetric distribution of intracellular organelles (e.g. apically oriented Golgi stacks), cytoskeleton arrangement (e.g. enrichment of filamentous actin in subapical zone), and pools of other intracellular proteins.
**Composition and apical sorting**

The apical domain faces varying, often harsh, environments and needs to protect itself from such a hostile exterior. By selective inclusion of lipids, proteins and carbohydrates, the apical domain has to protect the cell from the hostile exterior, yet needs to be flexible to allow passage of nutrients and, in some cell types, allow secretory capacity. The outer leaflet of the apical plasma membrane domain is enriched in glycosylated proteins, glycosphingolipids, and sterols. Such a detergent-resistant membrane domain phase-separates into sub-domain called **lipid rafts** which help cluster apical proteins and certain lipids (Simons, 1998). An ordinary mammalian plasma membrane contains 20% cholesterol, 15% sphingomyelin, and 5% glycolipids but, in polarized kidney epithelia, the combined cholesterol plus sphingomyelin proportion goes up to 60%. This lipid raft composition varies between different epithelia, e.g. intestinal epithelium have modest cholesterol proportion but >30% raft-promoting glycolipids (Gert Hansen, 2006). Such compositional differences allow for a robust lipid platform which in one case promotes ion exchange while in another case helps counter the action of detergent-like bile salts, without compromising the dynamic nature of both membranes. The unusual high proportion of lipid rafts makes the apical domain a continuous large lipid raft with patches of non-raft embedded (Schuk and Simons, 2004). This apical raft domain is replenished by exocytic delivery of Golgi-derived vesicles rich in lipid-micro domains. Lipid rafts help cluster proteins for apical delivery and shape apical sorting signals. Clustering, together with the association of proteolipids stimulates larger oligomers causing phase separation and efficient formation of apical directed vesicles (Schuk and Simons, 2004; Rescher and Gerke, 2004; Klemm et al, 2009). Lipid rafts are associated with the outer leaflet of the apical bilayer. The inner leaflet is enriched in **phosphatidylinositides**, especially phosphatidyl inositol-4, 5 bisphosphate (PIP2) which is continuously being generated by cytosolic kinases (such as phosphatidyl inositol-5 kinase) or cytosolic phosphatases (such as PTEN). The basolateral membrane is in enriched phosphatidyl inositol-3, 4, 5 triphosphate (PIP3) generated by local activity of the inositide kinases (such as phosphatidyl inositol-3 Kinase). This lipid gradient of PIP2 and PIP3 in the inner leaflet is important in the maintenance of cell polarity. PIP2 is also a secondary messenger and, by binding to structural proteins under the apical membrane, acts as a polarized signaling lipid. Since the composition of the luminal membrane is essential for epithelial function, several redundant mechanisms co-exist making apico-basal sorting a robust mechanism for maintaining the apical membrane. A detailed overview of the apical membrane sorting and clustering signals was recently provided in a review by Rodriguez-Boulan in 2009.

**Retention of apical proteins**

Polarised trafficking contributes to the establishment of the polarity program and helps maintain different domain identities. There are various mechanisms to ensure selective accumulation of the proteins on the apical domain. Differential trafficking of integral membrane proteins to the apical surface is coordinated with their **selective sorting** at trans-
The delivered proteins are continuously endocytosed and recycled to the plasma membrane or sent into the lysosomal degradative pathway. Apical and basolateral recycling thus handle distinct cargoes which may temporarily mix in common recycling endosome (CRE). The route from the TGN to apical surface is not always direct. En route Apical Recycling Endosomes (ARE) can act as sorting stations for post-Golgi and post-endocytic vesicles. Some membrane proteins are not sorted by but selectively retained on the apical membrane. Mechanisms include: i) binding to PDZ-domains exemplified by binding of proteins to NHERF/EBP50, ii) actin-linked proteins, like villin, or iii) binding to specific lipid moieties such as the local capture of ezrin by PIP2. Other mechanisms include displacement of membrane proteins from the basolateral surface by endocytosis and further apical transcytosis. (van IJzendoorn, 1999).

Stability of the apical domains also relies on the lateral diffusions in the membrane. The lateral diffusion of proteins and lipids residing in the outer leaflet of the membrane bilayer is limited by the presence of intra-membranous molecular fencing by tight junctions (Hoekstra, 2003). The TJ-associated proteins like cingulins, zona-occludens form macromolecular platforms linking the membranes to apical signaling machinery. Claudins and junctional adhesion molecules connect membranes from adjacent cells and reduce the paracellular flow of molecules. Localization of the conserved aPKC-PAR polarity complex at the TJ suggests the close knit association of tight junction formation and signal transduction driving polarity (reviewed by B. Margolis in 2006).

In conclusion, the apical plasma membrane is a lipid raft-rich domain displaying unusual structural aspects and distinct macromolecular composition which is generated by dynamic sorting, trafficking and local retention by membrane proteins. With the advent of newer biochemical and genetic tools, we are beginning to understand the organizational complexity and unique structural aspects of the apical plasma membrane.
**Microvilli – The apical boundary**

The apical membrane is divided into sub-domains. One of the unifying features of polarized epithelia is the formation of distinct protrusions of 80nm thickness and 1-2µm long forming the apical domain called microvilli (a.k.a **brush border**). Such organized, actin-rich, membrane protrusions substantially increase the absorptive surface area of the cells, especially in the liver, kidney and intestinal epithelia. Each microvillus is supported at its roots by a dense packing of cytoskeleton called the terminal web (TW). The TW and protrusions are enriched in cytoskeletal elements like intermediate filaments, microfilaments, and associated motor proteins. This unique structure of MV has been studied for a long time in terms of its composition and structural features but there is relatively little known on the signaling and trafficking mechanisms which lead to the formation of the MV. Some of the recent developments on the individual contributions of (1) Cytoskeletal proteins, (2) Cytoskeleton-membrane linker proteins, 3) intra-cellular trafficking pathways and how these contribute to microvilli development in a coordinated fashion are reviewed in this part of the chapter.

a) **Cytoskeletal elements**

MV are supported by parallel arrays of actin filaments that create a paracrystalline arrangement: the **actin bundle**. Individual actin filaments within the bundle are unipolar with their barbed, plus-ends at the top of the MV. Actin filaments constantly polymerise at the tip of each bundle (Mooseker, 1975; Revenu et al, 2012; McKnight and Brown, 2012). Specific proteins bundle and crosslink individual actin microfilaments to help form regular, tight, parallel arrays. This is needed to form uniform arrays seen in membrane protrusions like filopodia, bristles, stereocilia, rhabdomeres, and MV. Three important actin-bundling proteins, espin, plastin-1 (or fimbrin), and villin, are present in the MV. Absence of espin as seen in Jerker mice as well as mutations in the human espin gene cause autosomal recessive deafness and vestibular dysfunctions possibly by destabilizing actin in stereocilia (MV-like structures in the inner ear) (Donaudy F. et al, 2006). Surprisingly, espin knock-out mouse showed no effects in enterocyte BB formation (Sekerkova and Bartles, 2011). Villin, an actin-capping / actin-bundling protein present mainly in epithelia which have dense MV, when over-expressed in fibroblast-like cells, induces tufts of MV structures (Freiderich E, 1989); but a villin knockout mouse yielded no significant alteration of the MV structure (Pinson et al, 1998). Surprisingly, the triple knockout mouse of villin, espin, and plastin-1 also showed MV, albeit reduced in length, with disbundled actin. The actin bundlers seemingly work together in a concerted fashion and contribute only in the MV length but not initiation, thus explaining the rather mild effect of their individual depletions. As yet unknown robust actin nucleation signals and actin-linker proteins (myosins, ERMs, membrane-bending proteins, formins) must guide the initial arrangement of longitudinal actin filaments to make the membrane protrusion (Revenu et al, 2012).

In addition to actin, **Intermediate filaments (IF)** form a thick periluminal network below the apical membrane which connects the terminal web to the adherence junctions.
Mutations in intermediate filament-organising protein IFO-1 in *C. elegans* model leads to dis-organization of apical microvilli as well as loss of epithelial integrity (Carberry et al, 2012). Keratin-19 interacts with Ca$^{2+}$-dependent lipid binding protein Annexin-II, the knockdown of which hampers microvilli development (Hein et al, 2011). The IF component, Keratin-8, binds ezrin, an ERM-protein, in its inactive form and thus regulates microvilli actin assembly. Keratin-8 also recruits phosphoinositide-dependent kinase-1 (PDK1) which activates the polarity complex kinase aPKCiota which, in turn, is important for enterocyte brush border (BB) (Wald et al, 2005; Salas et al, 2012). Moreover, loss of actin-bundling protein plastin1 caused disruption of the apical IF network leading to shortened intestinal microvilli (Grimm-Gunter, 2009).

Desmosomal proteins, including desmoplakins and plakoglobins connect IFs to adhesion junctions and control the length of the MV in intestinal epithelium, but independent of their role in IF organization or terminal web assembly and possibly by signaling towards actin reorganization (Sumigray, 2012). Even IF keratin-binding protein Albatross, which connects the apical IFs with adhesion junction proteins, binds polarity complex protein Par3 and thereby regulates epithelial polarization does not play a role in MV organisation (Sugimato et al, 2008). In spite of the enrichment of various IF proteins in the terminal web and with their established roles as scaffolding proteins, how they contribute to signaling in the brush border formation is still unclear.

The dense cytoskeleton network underlying the MV provides a dynamic platform for the regulation of MV organisation and maintenance. Cytoskeleton too is in contact with the apical membrane and thus shapes membrane architecture. Such proteins that facilitate cytoskeleton-membrane interactions are linker proteins, some of which are reviewed in the next section.

b) Cytoskeleton-membrane linker proteins

The arrays of actin bundles are linked to the apical plasma membrane by various actin motor proteins including myosins. The head domain of myosin 1a, plus-end-directed motor, binds actin while its tail domain interacts with MV membrane lipids to induce tension on the protruded membrane so powering movement towards the MV tips. This outward push on the membrane has been studied by use of optical tweezers and live imaging on isolated BB (Tyska, 2007). The tread milling of actin filaments connected by myosin 1a propels the membrane over actin and causes shedding of microvillus membranes into the lumen environment in the form of micron sized vesicles. Myosin 2, which forms an actomyosin contractile ring along the cell cortex and contributes to brush border dynamics by connecting actin rootlets, has no role in the outward force generated on MV. The knockout mouse for myosin 1a has modest effect on BB density but causes irregular MV packing, disorganization, and length. The modest phenotype on myo1a knockout BB is possibly a result of myosin complementation. Several related myosins like Myo1b, Myo1c, and Myo1d are ubiquitously expressed in various cell types where they localize to the
MV tips. In fact, Myosin 1c specifically redistributes to the apical domain from basolateral domain to compensate for Myosin 1a (Benesh et al, 2010). Apical resident proteins such as CFTR that are involved in maintaining steep ion gradients are also dependent on this myosin (Ameen, 2012). Shotgun mass spectrometry on isolated brush borders identified, in addition to membrane bending machineries and adhesion proteins, low abundance myosins like myosins Va, Vb, VI, VIIa, VIIb and even previously undetected myosin XV (important in stereocilia) (McConnell et al, 2011). Myosin VI, the only minus-end-directed actin motor is also implicated in hearing loss in humans and mouse model (Snell’s Waltzer mouse which has a mutation in the MYO6 gene causing loss of motor function) due to irregular organization of stereocilia formation. Myosin VI is expressed throughout the intestine and is specifically located in the inter-microvillar apical membrane and subapical recycling endosomes. Snell-waltzer mouse also shows terminal web defects and disorganized fused MV in the intestine. The dependence of minus-end-directed myosin VI on activity of plus-ended-directed myosin 1a in the same niche shows the functional synergy between two motors and maintaining the protein composition of the apical membrane. Also myosins Va and 1e are up-regulated in these cells suggesting functional redundancy between various myosins. (Hegan et al, 2012; Buss et al, 2004; Apodaca, 2007; Benesh et al, 2010). A related motor protein myosin Vb, a regulator of recycling endosomes, is highly expressed in intestinal epithelium and mutations in the MYO5B gene also lead to disorganized villi and almost complete loss of MV in enterocytes of patients diagnosed with microvillus inclusion disease. Thus, the presence of myosins known for their roles in vesicular trafficking, indicates the overlapping and compensatory trafficking mechanisms which together lead to a healthy brush border organisation (Ameen, 2012).

Another major membrane-actin linker family present in the BB proteome is Ezrin-Radixin-Moesin (ERM) family of proteins. These proteins have an N-terminal membrane binding FERM (4.1, Ezrin, Radixin, Moesin) domain and a C-terminal actin binding domain (C-ERMAD) creating a functional link between apical membrane and the underlying cytoskeleton. Their expression is regulated in a developmental and tissue-specific manner and varies within different cell types. Many epithelial cells express ezrin, endothelial and neuronal cells prefer moesin, while radixin is majorly produced by hepatocytes (Fehon et al, 2010). Initially in an inactive state, FERM and C-ERMAD domains in ERM proteins are bound to each other yielding a closed conformation to the protein. In a concerted process of binding to the apical lipid moiety (mainly PIP2) and phosphorylation by kinases at Thr567 residue for ezrin (Radixin Thr564, Moesin Thr558), the intermolecular interaction is disrupted (Fievet et al, 2004; Matsui et al, 1998). This unmasks an alternate conformation which allows the direct interaction of C-ERMAD to actin and FERM to membrane / membrane-associated proteins (Figure 2).

Ezrin deficient mice show severe abnormalities in the shape of intestinal villi, fail to thrive, and die within 3 weeks (Saotome et al, 2004). In addition, intestinal absorptive cells in ezrin knockout mice cause disorganization of the actin terminal web and show short, sparse and immature MV (Louvard et al, 1992; Saotome et al, 2004). Ezrin knockout mice lose the
intestinal epithelial organization but do not manage to completely lose the brush border. The binding partners of ezrin CD43/44, ICAM1/2, NHE1/3, Syndecan 2, beta-dystrophin on the apical membrane and cytosolic adaptor proteins like EBP50, E3KARP, and others have a significant contribution in the disruption of the FERM-C-ERMAD interaction (Terawaki et al, 2006). Knockdown or introducing mutation that causes ezrin-binding defect in EBP50 leads to MV defects as well (Hanono et al, 2006). EBP50 in turn binds various other apical transmembrane proteins with their PDZ domains like NHE3, CFTR, Crumbs-3, podocalyxin, PDGFR, etc. Phosphorylation of EBP50 by PKC is necessary to augment the binding of PDZ domains to their respective ligands (Garbett et al, 2010). Thus ezrin and its binding partners provide complex networks to connect apical membrane and cytoskeleton driving BB formation.

Figure 2
Illustration displaying important apical proteins such as ICAMs, CD44, EBP50, NHERF (EBP50) and its binding partners NHE3, Crumbs-3, that regulate the dynamics of microvilli organization by influencing the localization and activation of ezrin, one of the key membrane-cytoskeleton linker proteins present at the brush border.

Ezrin activation occurs at the apical membrane by the recruitment of local threonine kinases (Fievet et al, 2004). The kinases that activate ezrin are diverse and have been the topics of recent studies. Earlier, Matsui et al (in 1998) showed Rho-kinases to play a role in phosphorylating ezrin in the apical domain. But this was questioned later (Matsui et al, 1999) and several other ERM kinases have been proposed in various cell models. Myotonic dystrophy kinase related cdc42-binding kinase (Nakamura et al, 2000), isoforms of protein kinase C (Pietromonaco et al, 1998) in fibroblasts, Ste20-like MST4 kinases, atypical protein kinase C and Akt2 (Ten klooster et al, 2009; Fidalgo et al, 2012, Wald et al, 2008, Shiue et al, 2005) in epithelial cells, and Lymphocyte-oriented kinase (LOK) in lymphocytes (Belkina et al, 2009). Recently, Vishwanathan et al (in 2012) have shown that in Jeg3 cells, Ste-20 like kinase (Slk) and LOK localize and activate ezrin at the microvillar tips. This form travels retrograde to the base to be eventually inactivated by cytoplasmic phosphatases ensuring a constant cycle of phosphorylation-dephosphorylation of ezrin. Too much phosphorylation causes its redistribution to the basolateral side, too little causes its mislocalization to the cytoplasm. Ezrin phosphocycling contributes to its apical localization and could be one of the important factors that make MV dynamic (Vishwanathan et al, 2012). The Presence of a handful of such kinases that are involved
in activating ezrin and regulating BB development could reflect variability in cell models as well as spatio-temporal differences in ezrin activation events at sub-domains along the MV. Studying how these kinases traffic to the MV would be useful to understand intra-microvilli dynamics and downstream processes.

An important gap in our understanding of BB development is how BB development is controlled by intracellular signaling. One might imagine a linker plus a scaffolding platform which organizes a macro-molecular signaling network in the apical niche. The molecular cues which originate from such ezrin complexes are yet to be determined. Ezrin regulates activity of Rho GTPase possibly using RhoGAP activity of its binding partners (Nadrin, ARHGAP18, PLEKHG6, RhoGDI) (Garbett et al, 2013). The intestines of ezrin KO mice show upregulated Rho activity affecting apical membrane rigidity (Casaletto et al, 2011). Other ERMs are known to contribute to membrane rigidity as shown by the blebbing phenotype in mitotic cells downregulated for moesin (Kunda et al, 2008). Apart from scaffold proteins like EBP50, ezrin also exerts its downstream signaling via actin capping factors like Epidermal growth factor receptor substrate 8 (Eps8). Ezrin-Eps8 interaction stimulates the bundling and capping activities of Eps8 and Eps8-like proteins during the initial budding of the BB (a basis of the “tip complex” proposed by in Mooseker in 1982 (Zwaenepoel et al, 2012). This is in line with the fact that Eps8 knockout mice show defects in fat absorption, possibly due to shorter MV length (Tochetti et al, 2010).

ERM proteins have thus emerged as important but not sufficient components of the apical BB assembly machinery and their phosphocycling, trafficking and downstream signaling regulate brush border development.

c) Uncoupling brush border signaling from polarity

Establishment of polarity is an underlying factor that stimulates MV formation. The polarized sorting and trafficking in time and space facilitates MV formation by providing the necessary machinery. Recently, several molecular links have emerged that define pathways that connect as well as disconnect the two. These include several small GTPase from the family of Arf, Rab, Ras, and Rho GTPases responsible for vesicular trafficking. GTPase Cdc42, belonging to the Rho GTPase family, is a critical component of epithelial cell polarity and has a role in cell differentiation and tissue morphogenesis. Its depletion (or over expression of a GTP-locked mutant) affects the permeability of intestinal epithelium, disturbs polarity (presumably by affecting the PAR-PKC-Cdc42 complex) and results in occasional cytoplasmic vacuoles that contain microvilli. Cdc42 displays a functional interaction with another GTPase, Rab8a, which is a regulator of apical recycling endosome network. This molecular cross talk controls each others’ GTPase activities to generate healthy brush border (Sakamori et al, 2012; Melendez et al, 2013). An important link is the protein EPI64 (Figure 3A), which connects MV assembly and apical endosomal machinery by binding both, EBP50 and GTP-Arf6 (PIP2-dependent regulator of endocytosis and membrane recycling). GDP-Arf6 or EBP50 mutants that are unable to functionally couple with EPI64 give rise to a reduced number of MV and the appearance of actin-rich vacuoles
in the cytoplasm (Hanono et al, 2006). Moreover, EPI64 displays RabGAP activity towards GTP-rab8a, a regulator of polarized trafficking. By reducing levels of GTP-Rab8a, EPI64 may limit the apical membrane recycling and stimulate actin nucleation / polymerization driving membrane protrusions (Hokanson and Bretscher, 2012). Thus, some of the cellular signaling pathways that coordinate epithelial polarity program and polarized protein trafficking also contribute in downstream events such as MV formation and maintenance.

Perturbing regulators of the endosomal system can also disturb epithelial polarity program (Weisz and Rodriguez-Boulan, 2009) including MV. Established cell lines like MDCK, CaCo2, LLC-PK1 and Jeg3 have been useful in investigating whether MV formation is an inevitable downstream effect of polarity. These cells spontaneously polarize and develop an apical BB after establishing cell-cell contact in monolayer cultures. LS174T-W4 is an intestinal epithelial cell model in which apical polarity and BB formation can be stimulated in single cells by doxycycline controlled over-expression of Strad-α. A molecular pathway has emerged where adaptor protein Strad-α triggers the export of kinase Lkb1, a polarity and oncogenic protein, from the nucleus to the cytoplasm allowing the formation of a tripartite complex of Mo25-Strada-Lkb1 at the cis-Golgi (Figure 3B). This may have a direct effect on Golgi trafficking by activating the Ste20-like kinase MST4 at the Golgi membranes. Activated MST4 translocates to the subapical zone of the cell via an unknown mechanism and phosphorylates the locally retained ezrin thereby helping brush border formation independent of apico-basal polarity establishment (Ten Klooster et al, 2009). This suggests that microvilli development program can be separated at the molecular level from general cell polarity program. This implies that a cell can maintain major aspects of polarity but independently of the characteristic BB organization of the apical surface.

This hypothesis is reinforced by the study on Rap2a which is involved in cell-cell adhesion and cell polarity. By use of cell-adhesion deficient LS174T-W4 cell line, Rap2a was shown to have a novel role in MV formation distinct from its established role in polarity. Rap2a is recruited to the apical plasma membrane by a series of signaling
cascades originating from the cleavage of PIP2 by phospholipase D (PLD1). The product, phosphatidic acid can then activate the GTPase cycle of Rap2a by binding PDZGEF. The PIP2-PDZGEF-Rap2a pathway recruits the effector TNIK (TRAF2 and NCK-interacting kinase) and induces actin reorganization. The signaling cascade merges with the above described Strada-Lkb1-Mo25 pathway to allow the phosphorylation of ezrin by recruiting its kinase MST4 (Gloerich et al, 2012). This road seems to be crucial to stimulate MV as tampering with the kinase activities or effectors leads to loss of BB by losing ezrin phosphorylation, but not loss of polarity. This underscores the uncoupling of pathways that drive BB from those that drive apico-basal polarity.

d) New perspectives on MV and open questions

Microvilli are traditionally considered as structures needed to expand the surface area of the cell. However, several recent studies have demonstrated that microvilli contribute to additional physiological functions.

Disruption of cell polarity is a major feature in the epithelial to mesenchymal transition leading to oncogenic transformation. Colon cancer cells often show inactivated myosin 1a by genetic or epigenetic mechanisms. Myosin 1a knockout mice have more aggressive tumors. The expression pattern of BB myosin 1a is an important prognostic marker for tumor progression (Mazollini et al, 2013). Surprisingly, another BB myosin, Myosin VI which together with Myosin 1a maintains the MV membrane tension, is also implicated in prostate cancer. Myosin VI is over-expressed in the medium-grade prostate cancers and is critical in maintaining the malignant properties of most diagnosed human prostate cancers (Dunn et al, 2006). This demonstrates that structural components of MV display tumor suppressor ability.

Another structural protein of MV also regulates migratory behavior of enterocytes during injury and wound healing. In addition to actin-binding, villin also displays an actin-severing function favored by calcium dependent phosphorylation at several tyrosine residues. Villin regulates actin plasticity that allows cells to migrate laterally within a monolayer. Actin severing deficiency in villin prevents the breakdown of microvilli and inhibits cell migration during wound healing. Thus without changing the polarity program, epithelial cells can control their migratory behavior simply by regulating MV dynamics. MV are not just a structural marker of polarity, but their components inhibit dedifferentiation in intestinal epithelium (Ubelmann et al, 2013). Another form of migration displayed by enterocytes is their movement from crypts to the tips of the villi. Their orientation relative to crypt-villus axis is critical in tissue morphogenesis and planar polarity. The critical microvillus component ezrin was shown to regulate spindle orientation during cell division. In ezrin knockout mice, the mitotic spindle angles relative to the crypt-villus axis was random while those in control mice were typically 30° (Casaletto et al, 2011). Such spindle orientation is important in maintaining the planar polarity during tissue homeostasis (Fraser et al, 2004; Slim et al, 2013) and is regulated by the BB components of the dividing cells in the intestinal crypt.
The BB of epithelia is responsive to external stimuli and thus is very dynamic. Membranes that are shed from the BB in the form of vesicles by myosin 1a controlled membrane-sliding mechanism also carry important enzymes on the external leaflet of their membrane bilayer. These vesicles can bind the gut microbes such as entero-pathogenic \textit{E.Coli} and reduce their adherence to the epithelial cell surfaces. The surface enzymes include alkaline phosphatase that helps detoxify the bacteria by de-phosphorylating the pro-inflammatory lipo-polysaccharides in the cell wall. In fact, the epithelial cells up-regulate shedding of micro-vesicles in the presence of pathogenic bacteria. Conditioning the lumen environment and helping in the innate immune response by generating vesicles seems to be an important role of microvilli. (McConnell et al, 2009). This phenomenon is common to many other cell types having MV, but their intra-cellular signaling is yet elusive. In addition, pore-forming toxins secreted by pathogenic bacteria in the gut injure the MV and apical membrane. Regulators of endocytosis and membrane recycling (GTPase rab5 and rab11) repair this insult by formation of a membrane patch and expulsion of the damaged MV to heal the injury to the apical membrane (Los et al, 2011). Such instances of a supply of membrane towards the injured MV region, or of shedding micro-vesicles from MV points towards a need for an uninterrupted supply of BB-targeted endo-membranes.

The mechanisms via which apical membrane dynamics regulates MV development are yet not well understood. In the secretory gastric parietal cells, upon stimulation by PKA agonists, a massive reorganisation of internal tubulo-vesicular membranes (TVM) and rapid increase in the apical surface area of these cells occur. The relatively short MV and canaliculi at the apical membrane are transformed into long MV, 5-10 fold increase in the surface area and the redistribution of the TVM from the cytoplasm to the apical membrane, thus facilitating the apical positioning of important anionic transporters (Forte and Zhu, 2010). Similarly, membrane fusion machinery such as Munc-18 and Syntaxins are upregulated at the apical membrane assisting fusion of endo-membranes and increase in surface area (Karvar et al, 2005; Liu et al, 2007). These TVMs also contain GTPase rab11, known for its dual role in apical membrane recycling and TGN-Apical membrane trafficking, to be upregulated and activity of which is required for stimulation of parietal cells. Activity of rab11a is shown to be required also in post-fertilized eggs of sea urchins to position membrane transporter to MV membrane (Whalen et al, 2012). Moreover, another regulator of apical membrane recycling, GTPase Rab8a, is implicated in regulation of MV. Depletion of GTPase Rab8a in a mouse model showed redistribution of apical peptidases and transporters to lysosomes, presence of microvillus inclusions as well as defects in apical MV elongation (Sato et al, 2007). Although the signaling pathways that coordinate endo-membrane trafficking and BB development is still unclear, the above examples suggest a strong molecular link with the apical endosomal system.

The involvement of membrane trafficking in MV development is exemplified in patients with microvillus inclusion disease (MVID), a rare and fatal trafficking disorder in the new born children. MVID patients carry mutations in the effector protein of GTPases rab11a (and rab8a), called myosin Vb, a motor protein that is important in regulating
the dynamics of recycling endosomes. These patients suffer from a persistent diarrheal disorder and a severe absorption problem. Mutations in MYO5B are associated with MV atrophy, cytoplasmic MV inclusions (Muller et al, 2008), and re-distributed rab11a in MVID enterocytes (Szperl et al, 2011). This suggests that a functional membrane trafficking pathway correlates with the organisation of a healthy BB. Whether and how signaling for BB assembly may be regulated by a flow of endo-membranes is yet unclear and has been investigated in this thesis.

**Conclusion**

To summarize, there are identifiable distinct links between the molecular events necessary for MV organization and apical protein trafficking machinery which maintains cell polarity. **Apical recycling endosomes** are the most recent and crucial link between polarity, MV dynamics and polarised vesicular trafficking. The AREs themselves have been crucial in contributing to polarized cell trafficking. **Microvillus inclusion disease**, (detailed in the next chapter) is a disorder characterized by defects in trafficking towards the apical membrane of intestinal epithelial cells. Because of the pronounced defect in the apical surface development, endosomal recycling system and loss of brush border, the study of the pathogenic mechanisms in microvillus inclusion disease provides a unique opportunity to gain insight into the molecular mechanisms that link intracellular trafficking and MV development and organization.
CHAPTER 1

References


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SCOPE OF THE THESIS

This thesis, titled “Pathogenic mechanism in microvillus inclusion disease – Focus on apical brush border”, investigates the cellular and molecular mechanisms that underlie the pathogenesis of MVID, a congenital diarrheal disorder. In this way, we aim to understand the mechanisms that shape the apical membrane landscape, with emphasis on the microvilli brush border. Since the disease causing gene leads to trafficking defects, we focused on the contribution of the endo-membrane system to the development and organization of the brush border and epithelial monolayer.

Chapter 1 introduced the concept of polarity, distinct features of the apical membrane and later dwells on the advances in the understanding of the molecular cues needed for microvilli development.

Chapter 2 is a review on microvillus inclusions disease from a clinical and basic research perspective. This chapter introduces the MVID patient registry and mutation database. The structure-function relationship of myosin Vb protein and the role of myosin Vb in cell biological processes are discussed. Mutations in other myosins which lead to diseases have also been compared.

Chapter 3 provides a mechanism for how myosin Vb loss-of-function leads to microvillar atrophy and not earlier observed defects in villi organization of MVID. A model as to how subapical endosomes, regulated by myosin Vb assemble machinery needed for brush border organization, is proposed.

Chapter 4 introduces a novel role for small GTPase Rac1 in microvilli organization. The apical localisation of Rac1, its role independent of cell-cell adhesion and polarity and irrespective of activity status in the brush border organisation is studied in this chapter.

Chapter 5 examines microvilli atrophy in two apparently unconnected congenital gastrointestinal disorders, MVID and FHL5, and how their paths meet at the apical recycling endosomes. The hypothesis that apical fusion machinery can contribute, directly or indirectly, in brush border organization is tested.

Chapter 6 touches on the role of paracellular permeability (leaky gut), one of the well-known contributing factors in causing secretory diarrhea, in MVID pathogenesis. Given the constitutive nature of diarrhea in the patients, inquiry into whether tight junctions contribute to the pathogenesis of the disease is made.