

Desmoplakin controls microvilli length but not cell adhesion or keratin organization in the intestinal epithelium

Kaelyn D. Sumigray^a and Terry Lechler^{a,b}

^aDepartment of Cell Biology and ^bDepartment of Dermatology, Duke University Medical Center, Durham, NC 27710

ABSTRACT Maintaining proper cell–cell adhesion in the intestine is essential for tissue homeostasis and barrier function. This adhesion is thought to be mediated by cell adhesion structures, including tight junctions, adherens junctions, and desmosomes, which concentrate in the apical junctional region. While clear roles for adherens and tight junctions have been established in simple epithelia, the function of desmosomes has not been addressed. In stratified epithelia, desmosomes impart mechanical strength to tissues by organizing and anchoring the keratin filament network. In this paper, we report that the desmosomal protein desmoplakin (DP) is not essential for cell adhesion in the intestinal epithelium. Surprisingly, when DP is lacking, keratin filament localization is also unperturbed, although keratin filaments no longer anchor at desmosomes. Unexpectedly, DP is important for proper microvillus structure. Our study highlights the tissue-specific functions of desmosomes and reveals that the canonical functions for these structures are not conserved in simple epithelium.

Monitoring Editor
Thomas M. Magin
University of Leipzig

Received: Nov 16, 2011
Revised: Dec 28, 2011
Accepted: Jan 6, 2012

INTRODUCTION

Desmosomes are essential cell–cell adhesion structures that resist mechanical stress and provide strength to tissues. These tasks are accomplished by robust linkage of the intermediate filament cytoskeleton to the dense intercellular junction. The desmosome links two cells via extracellular interactions between desmosomal cadherins on neighboring cells. The cytoplasmic tail of each cadherin is linked to several proteins, including the armadillo family protein plakoglobin (γ -catenin). Plakoglobin then binds the plakin family member desmoplakin (DP), which provides the ultimate link between the desmosome and the intermediate filament cytoskeleton (Stappenbeck and Green, 1992; Bornslaeger *et al.*, 1996).

Loss of DP results in early embryonic lethality due to extraembryonic defects (Gallicano *et al.*, 1998). Rescue of DP in extraembryonic tissues allows embryos to develop until shortly after gastrulation, at which point they die due to defects in the heart, skin, neuroepithelium, and vasculature (Gallicano *et al.*, 2001), each of which experiences mechanical stress, highlighting the importance of desmosomes and their attachment to intermediate filaments in these tissues. When DP is lost only in mouse epidermis, the mice die perinatally due to extensive intercellular separations, blistering, and loss of barrier activity (Vasioukhin *et al.*, 2001). In humans, alterations in the DP gene lead to a range of diseases, including blistering of the skin, cardiac myopathies (Thomason *et al.*, 2010), and in the case of severe loss of function, death (Jonkman *et al.*, 2005).

While the essential function of desmosomes in the stratified epithelia and cardiomyocytes is well defined, their roles in other tissues, especially simple epithelia, remain unclear. The intestinal epithelium is a highly polarized columnar epithelium with junctional complexes localized to a subapical region of the cell; the tight junction is most apical, followed by the adherens junction, and finally the desmosome. The intestinal epithelium is exposed to several sources of physical forces. These include villus motility, in which the villi spontaneously and repetitively contract (Womack *et al.*, 1987), peristalsis, and shear stress from the contents of the

This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-11-0923>) on January 11, 2012.

Address correspondence to: Terry Lechler (terry.lechler@duke.edu).

Abbreviations used: DP, desmoplakin; DP cKO mice, *Villin-Cre; DP fl/fl* mice; EGTA, ethylene glycol tetraacetic acid; KO, knockout; PBS, phosphate-buffered saline; PFA, paraformaldehyde; sulfo-NHS-biotin, sulfo-*N*-hydroxysuccinimide-biotin; WT, wild type.

© 2012 Sumigray and Lechler. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (<http://creativecommons.org/licenses/by-nc-sa/3.0>).

“ASCB®,” “The American Society for Cell Biology®,” and “Molecular Biology of the Cell®” are registered trademarks of The American Society of Cell Biology.

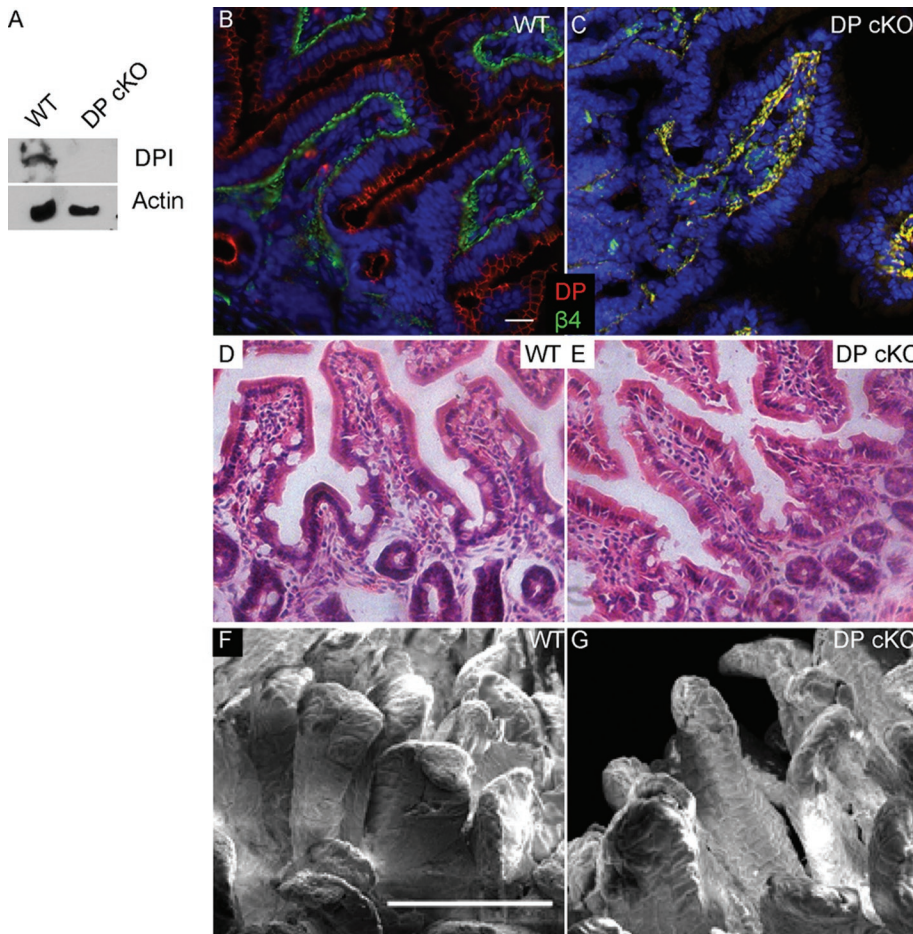


FIGURE 1: DP cKO intestine is indistinguishable from WT. (A) DP protein is lost in DP cKO intestinal epithelial cells. β -actin was used as a loading control. (B and C) Loss of DP (red) protein by immunofluorescence in tissue sections. The basement membrane is marked by $\beta 4$ integrin (green). Images are shown as maximum-intensity projections. (D and E) Hematoxylin and eosin staining of WT (D) and DP cKO (E) small intestine. (A–D) Scale bar: 20 μ m, as shown in (B). (F and G) Scanning electron micrograph of villi in WT (F) and DP cKO (G) small intestine. Scale bar: 200 μ m.

intestinal lumen. In each of these cases, the intestine must resist the forces to maintain an intact epithelial sheet.

The junctional complex anchors cytoskeletal networks to the subapical membrane, at which they integrate to form the terminal web. The terminal web is important in maintaining and supporting the microvilli that extend from the apical surface of the cell (Karagiorgos and Ready, 2004; Saotome *et al.*, 2004). Significantly, keratins, which are tethered to the desmosome, have been implicated in organizing the subapical region of the cell and as important factors in cell polarity and proper organization of the apical region of cells (Salas *et al.*, 1997; Ameen *et al.*, 2001). Loss of keratin 8, the major type II intermediate filament present in the intestinal epithelium, resulted in mislocalization of several apical membrane proteins (Ameen *et al.*, 2001). In another study, cultured cells with decreased levels of keratin 19 showed defects in actin organization, including a decrease in the number of microvilli, microtubule organization, and apical protein localization (Salas *et al.*, 1997). These results demonstrate the importance of the keratin cytoskeleton in cell polarity and proper apical domain maintenance. Whether the attachment of keratin to the desmosome is critical for these functions is unclear.

How desmosomes contribute to both cell–cell adhesion and cytoskeletal organization in the small intestine is not known. To

address this question, we made intestinal epithelium–specific DP knockout (KO) mice using the *Villin-Cre* transgene (Pinto *et al.*, 1999). Our data reveal a role for DP in maintaining the shape and length of actin-rich microvilli. Surprisingly, however, DP is dispensable for tissue integrity, cell–cell adhesion, and the apical accumulation of keratin.

RESULTS

To determine the role of DP in the simple epithelium lining the small intestine, we mated *DP fl/fl* mice with *Villin-Cre* transgenic mice to create *Villin-Cre; DP fl/fl* mice (hereafter referred to as DP cKO mice) and then backcrossed them for at least four generations into the C57BL/6 background. We confirmed the loss of DP in the intestinal epithelium by Western blotting and immunofluorescence (Figure 1, A–C). DP is apically enriched in the epithelium of wild-type (WT) mice, and this was completely lost in DP cKO mice. Loss was specific, as DP was still detected in endothelial and other epithelial tissues (unpublished data).

The DP cKO mice were indistinguishable from WT littermates and were viable and fertile. The life span of DP cKO mice was not altered, and there were no changes in growth rate, body weight, or intestine length. The morphology of the DP cKO intestine was similar to WT intestine, with no notable difference in the villus/crypt architecture (Figure 1, D and E). Analysis by scanning electron microscopy also revealed regular villi that appeared very similar to WT intestine (Figure 1, F and G). Furthermore, differentiation did not seem to be affected, as the number of goblet cells was comparable between WT and DP cKO (unpublished data). Finally, the level of cell proliferation was not changed by loss of DP.

To further define the role of DP in the intestine, we began by examining the status of cell adhesion structures. In the epidermis, DP is not required for the membrane localization and formation of the desmosomal plaque (Vasioukhin *et al.*, 2001). This is also true in the intestinal epithelium. Plakoglobin and plakophilin 2 localization, as assessed by indirect immunofluorescence, was normal in the DP cKO mice, concentrating at the apical region of the cell (Figure 2, A–D). Visualization of adherens junctions with E-cadherin and tight junctions with ZO-1 revealed no changes in the apical junctional complex and revealed contiguous staining of the cells (Figure 2, E–H). These data indicate that DP is not required for cell–cell adhesion in the epithelium of the small intestine. This is in sharp contrast to the DP cKO epidermis, in which blisters and intercellular separations are common (Vasioukhin *et al.*, 2001). We confirmed that the barrier function of the epithelium was intact in DP cKO intestine by injecting sulfo-*N*-hydroxysuccinimide-biotin (sulfo-NHS-biotin) into the lumen of the intestine. When the barrier is functional, the biotin cannot move past the tight junction; however, when the barrier is compromised, biotin can diffuse past the tight junction and move down lateral membranes, as seen in WT intestine treated with 2 mM

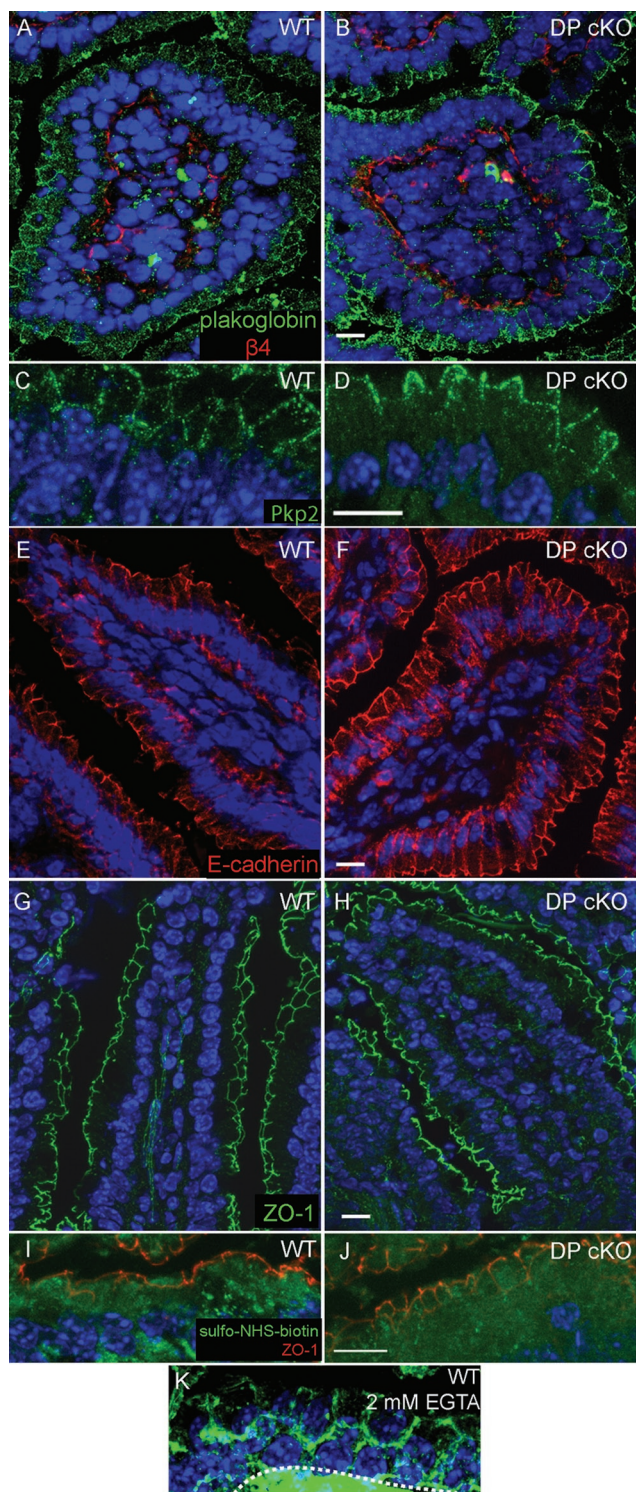


FIGURE 2: DP is not required for cell–cell adhesion. (A–D) Desmosomal staining with plakoglobin (A and B, green) and plakophilin-2 (Pkp2; C and D, green) in WT and DP cKO intestine. β 4 integrin marks the basement membrane (red) in (A) and (B). (E and F) Localization of the adherens junction protein E-cadherin (red). (G and H) Localization of the tight junction protein ZO-1 (green). (I and J) Barrier assays in WT (I) and DP cKO (J) intestine. Biotin (green) is excluded from the lateral membrane in both WT and DP cKO. ZO-1 (red) marks the tight junctions. (K) WT intestine was treated with 2 mM EGTA to disrupt cell–cell junctions. Biotin (green) is at lateral membranes. The basement membrane is marked by a white dotted line. All images are shown as maximum-intensity projections. All scale bars: 10 μ m.

ethylene glycol tetraacetic acid (EGTA) to disrupt cell–cell adhesion (Figure 2K). We did not observe any lateral membrane localization of biotin in either the WT or DP cKO intestine (Figure 2, I–J), demonstrating that DP is not required for barrier function of the intestinal epithelium. Consistent with this, no signs of inflammation due to bacterial infection or the ability of the bacteria to cross the barrier were noted.

Because DP is required for keratin filament anchorage to the desmosome in the epidermis (Vasioukhin *et al.*, 2001) and cultured cells (Green *et al.*, 1992; Stappenbeck and Green, 1992), we examined keratin filament localization in DP cKO intestine. Ultrastructural analysis revealed that desmosomes were present in their proper subapical location at each cell–cell junction in DP cKO mice, confirming our immunofluorescence data (Figure 3, A–B). However, unlike in the WT intestine, keratin filaments were no longer in contact with the desmosome (Figure 3, C–D). We did not observe any desmosomes with robust filament attachment in the DP cKO intestine. Surprisingly, however, keratin filament organization in DP cKO intestine was indistinguishable from keratin organization in the WT intestine at the light microscope level. This was true for keratins 7, 8, 17/19, and 18 (Figure 3, E–J; unpublished data). Despite its lack of attachment to the desmosome, the keratin network was enriched in the apical region of the intestine, presumably incorporated into the terminal web. Therefore, although DP is required to anchor keratin filaments, it is not required to localize the network to the apical region of the cell.

In addition to its ability to bind intermediate filaments, DP is required for microtubule organization in the epidermis (Lechler and Fuchs, 2007). To determine whether the same is true in the small intestine, we examined α -tubulin staining in the WT and DP cKO mice. In WT and DP cKO enterocytes, microtubules were organized along the apicobasal axis (Figure 4, A and B). Therefore, unlike the epidermis, the small intestine does not require DP to organize its microtubules. To determine why there might be a difference in requirements for microtubule organization in the intestine compared with the epidermis, we examined the localization of other proteins implicated in epidermal microtubule organization. In the differentiated cells of the epidermis, DP recruits a group of centrosomal proteins to the cell cortex (Lechler and Fuchs, 2007; Sumigray *et al.*, 2011). These proteins (including ninein and Lis1) have been proposed to act downstream of DP to organize microtubules at the cell cortex, and at least one of these proteins is required for cortical microtubule organization (Sumigray *et al.*, 2011). Ninein localized to the apical region of enterocytes in WT intestine, consistent with a conserved function of desmosomes in its recruitment (Figure 4C). In contrast, ninein was cytoplasmic in DP cKO enterocytes (Figure 4D). Similarly, Lis1 was found at cell–cell junctions in WT enterocytes and in a large cytoplasmic pool (Figure 4E). However, in DP cKO villi, Lis1 was not junctional, but was localized to the apical region of the cell (Figure 4F). Therefore the recruitment of centrosomal proteins to the desmosome is a function of DP that is conserved in different tissues. However, while this recruitment is important for microtubule organization in the epidermis, it is dispensable in the intestine.

DP has also been implicated in actin cytoskeleton organization (Vasioukhin *et al.*, 2001). To determine whether loss of DP results in actin organization defects, we examined phalloidin staining of intestinal sections. Phalloidin strongly labels the actin-rich microvilli that make up the brush border, as well as the underlying terminal web and the lateral walls. In sections, we saw that phalloidin labeled a thinner region of the apical surface of DP cKO intestine compared with WT intestine (Figure 5, A and B). The lateral staining was not changed, suggesting a specificity for the requirement of DP in the

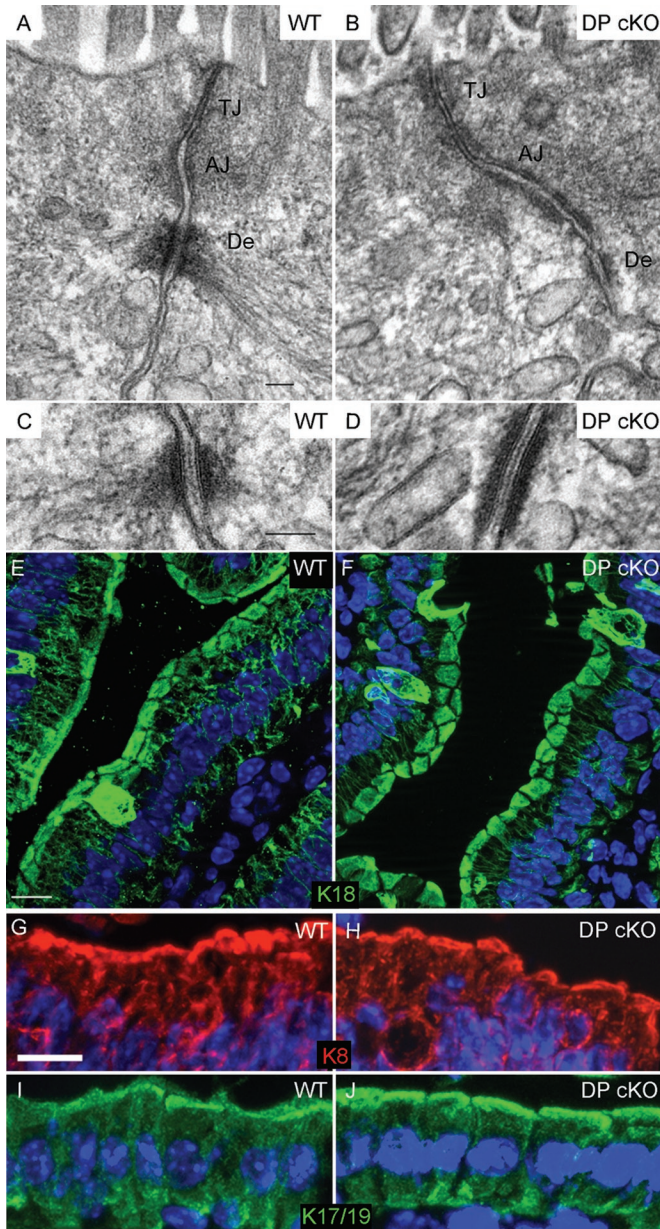


FIGURE 3: DP anchors, but does not localize, keratin filaments. (A and B) Transmission electron micrograph of the apical junctional complex in WT (A) and DP cKO (B) intestine. TJ, tight junction; AJ, adherens junction; De, desmosome. Scale bar: 100 nm. (C and D) Transmission electron microscopy of a desmosome in WT (C) and DP cKO (D) intestine. Note the loss of keratin filament attachment in DP cKO. Scale bar: 100 nm. (E and F) Immunofluorescence of keratin 18 (green) in WT (E) and DP cKO (F) villi. Keratin is enriched apically in each sample. Scale bar: 10 μ m. The images are shown as maximum-intensity projections. (G and H) Immunofluorescence of keratin 8 (red) in WT (G) and DP cKO (H) villi. (I and J) Immunofluorescence of keratin 17/19 (green) in WT (I) and DP cKO (J) villi. (G–J) Scale bar: 10 μ m, as shown in (G).

actin organization in the terminal web or brush border. To further examine the structure of the microvilli, we performed transmission electron microscopy. Although DP cKO enterocytes maintained microvilli on their apical surfaces, the microvilli were shorter than WT microvilli (Figure 5, C–E). In addition, the microvilli in DP cKO intestine were often irregularly shaped and vesiculated (Figure 5E). We

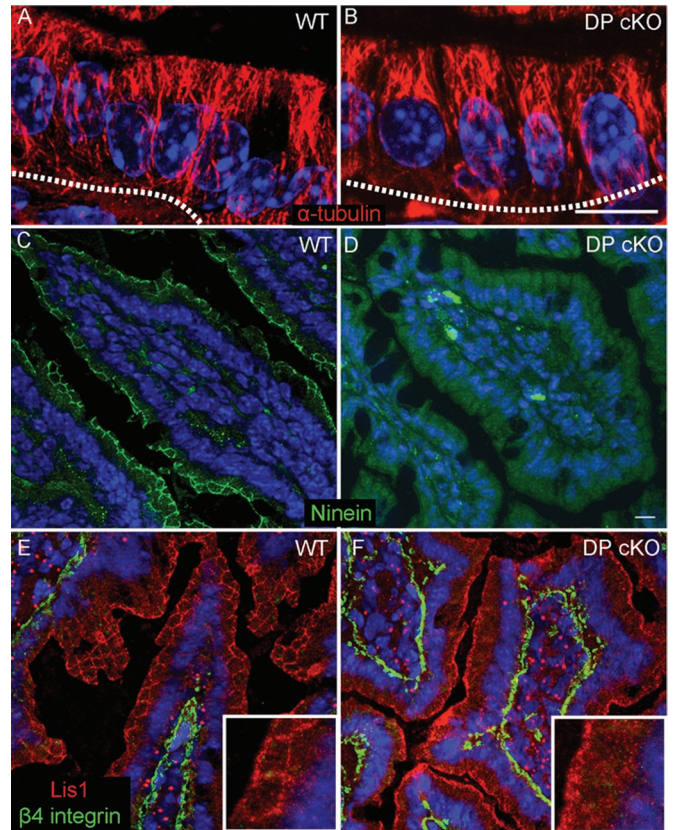


FIGURE 4: DP is not required for microtubule organization. (A and B) α -tubulin (red) staining reveals apicobasal organization of microtubules in WT (A) and DP cKO (B) intestine. The basement membrane is marked by a white dotted line. Scale bar: 10 μ m. (C and D) Immunofluorescence staining of the microtubule-anchoring protein ninein (green). Ninein is junctional in WT villi (C) but cytoplasmic in DP cKO villi (D). (E and F) Lis1 (red) staining in WT (E) and DP cKO (F) villi. β 4 integrin (green) marks the basement membrane. (C–F) Scale bar: 10 μ m, as shown in (D). All images are maximum-intensity projections.

observed shortened and irregular microvilli in outbred mice as well. The change in length was not as severe in outbred mice at 3 mo of age, but it progressively worsened in 1-yr-old mice (unpublished data).

Defects in the brush border could be secondary to defects in the terminal web. We therefore examined the levels and localization of protein components of the terminal web in WT and DP cKO intestine. As seen by immunofluorescence staining, all of the terminal web components examined were similarly localized in WT and DP cKO intestine (Figure 6, A–L). Furthermore, as shown by Western blot analysis, the proteins were expressed at similar levels (Figure 6M). Ultrastructurally, the size of the organelle-free zone was slightly decreased in DP cKO intestinal cells (Figure 6N). These data suggest that there is no gross defect in the terminal web. However, the change in the organelle-free zone may indicate subtle organization problems that could underlie the brush border phenotype.

This study has revealed an unexpected role for DP in controlling microvilli architecture. Furthermore, DP is not required for proper cell–cell adhesion, tissue integrity, or keratin organization of the intestinal epithelium, the widely accepted canonical functions of the desmosome. Therefore desmosomes are important regulators of several cytoskeletal networks in diverse tissue-specific manners.

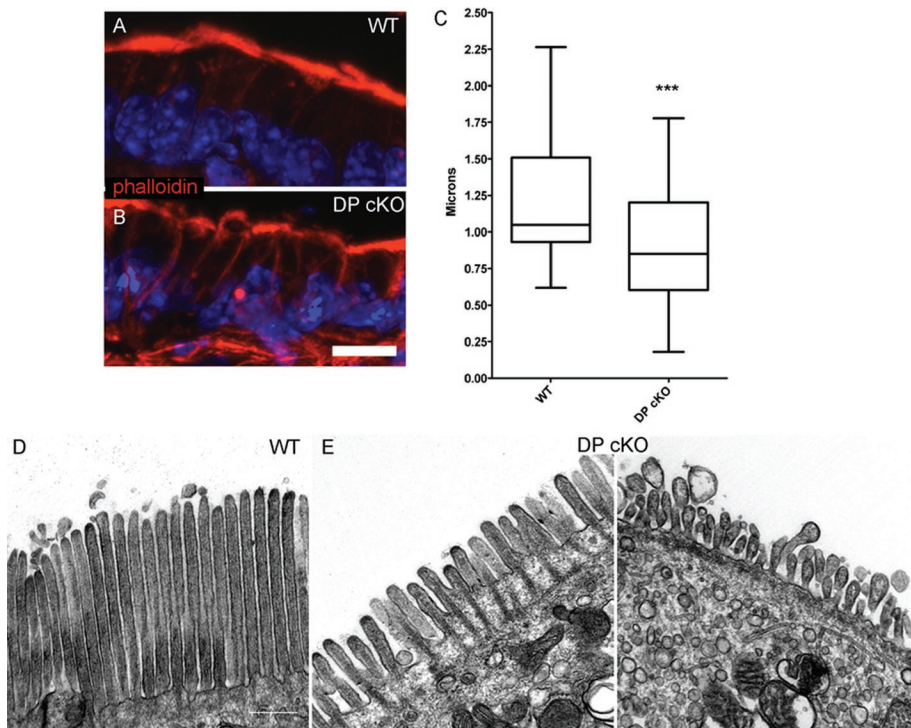


FIGURE 5: Loss of DP results in microvilli defects. (A and B) Phalloidin (red) staining on the apical surface of WT (A) and DP cKO (B) intestinal cells. Scale bar: 10 μ m. Images are maximum-intensity projections. (C) Box-and-whisker plot of microvilli length. The edges of the box represent the 25th and 75th percentiles, and the whiskers are the minimum and maximum. ***, $p < 0.0001$. (D and E) Transmission electron micrographs of the apical surface of WT (D) and DP cKO (E) enterocytes. The projections off the surface are microvilli. DP cKO intestine has short microvilli that are often misshapen (E). Scale bar: 500 nm.

DISCUSSION

DP forms a critical link between desmosomes and intermediate filaments and is necessary for the desmosome to provide strength to tissues undergoing mechanical stress. We have shown that the desmosome–keratin link is dispensable for tissue integrity and cell–cell adhesion in the intestine. We have, however, discovered an unexpected role for DP in the control of microvillar architecture.

In some tissues, desmosomes organize the intermediate filament cytoskeleton and maintain its proper localization within the cell. For example, in DP-null keratinocytes, keratin filaments do not form a robust cortical belt as they do in WT keratinocytes (Vasioukhin *et al.*, 2001). Furthermore, in DP-null embryos, the normally extensive keratin filament network that extended throughout the cytoplasm was reduced to keratin filaments found predominantly at the cell periphery (Gallicano *et al.*, 1998). Therefore, in other tissues, DP's association with the intermediate filament cytoskeleton is required for the proper organization of the intermediate filament network. This is not the case in the small intestine; although loss of DP disrupted keratin's association with the desmosome, the intermediate filament network remained largely undisturbed. Although we cannot rule out that other proteins can partially rescue keratin filament attachment to the desmosome, our data suggests that DP is the primary protein involved. Keratins still localized to the apical region of the cell, the point at which they were most likely incorporated into the terminal web, as in WT enterocytes. These data demonstrate that desmosomes are not responsible for proper positioning of the keratin cytoskeleton in the small intestine. The keratin cytoskeleton, however, is important in organizing the apical region of some cells, including enterocytes (Salas *et al.*, 1997; Ameen *et al.*, 2001). Based

on our data, this role of keratins must be independent of their ability to bind the desmosome, as we did not observe any polarity defects or mislocalization of proteins from the apical domain of DP cKO intestinal cells (unpublished data).

If not desmosomes, what mediates apical keratin localization? Several possibilities exist. Intermediate filament precursors have been shown to move along microtubules toward the cell periphery in other cell types (Pralhad *et al.*, 1998). If the same is true in the intestine, then movement of keratin particles to the apical surface would occur normally because microtubule organization is normal in the absence of DP. Once the keratin filament precursors arrive at their proper subcellular localization, they may be integrated into the existing keratin filament network. In addition, protein components of the terminal web, like plastin, can bind to both F-actin and keratin filaments (Grimm-Gunter *et al.*, 2009). Because plastin still localizes properly in the DP cKO intestine, it could recruit and/or maintain keratin filaments in the apical region. Additionally, the intermediate filament-binding protein plectin remains apically localized in the DP cKO intestine, and may mediate the localization of keratins to the subapical region of the cells. Furthermore, several apical membrane proteins can be found in a complex with keratin (Rodriguez *et al.*,

1994). They may play a role in maintaining the apical localization of the keratin cytoskeleton. Whether the keratin filament network is assembled elsewhere and then transported to the apical region of the cell, or whether the keratin filaments are assembled at their final subcellular localization is unknown.

Surprisingly, desmosomal attachment to keratin filaments is not required for maintaining cell–cell adhesion in the small intestine. This is the first reported case in which loss of DP does not result in cell–cell adhesion or tissue integrity defects. As previously mentioned, loss of DP in the epidermis results in severe blistering and intercellular separations, leading to perinatal death (Vasioukhin *et al.*, 2001). Similarly, human mutations in DP result in blistering disorders (Jonkman *et al.*, 2005; Thomason *et al.*, 2010). Complete loss of DP in the early mouse embryo (but not in the extraembryonic tissue) results in malformed neuroepithelium, heart, skin, and vasculature (Gallicano *et al.*, 2001); in these cases, the tissue often appeared collapsed. While the stress and forces that the intestine is exposed to may be less intense than those experienced by the epidermis or the beating heart, it is surprising that DP is not required for tissue integrity in the intestinal epithelium. It is presently unclear what cellular structures buffer the forces that the intestinal epithelium normally encounters.

Unlike in the epidermis, DP is not required for microtubule organization in the small intestine. The apicobasal array of microtubules was undisturbed in DP cKO intestine, even though DP is still required to recruit the microtubule-binding proteins ninein and Lis1. In the epidermis, this pathway is essential to organize microtubules at the cell cortex (Sumigray *et al.*, 2011). Although potentially active in the intestine, this pathway may be redundant, with additional

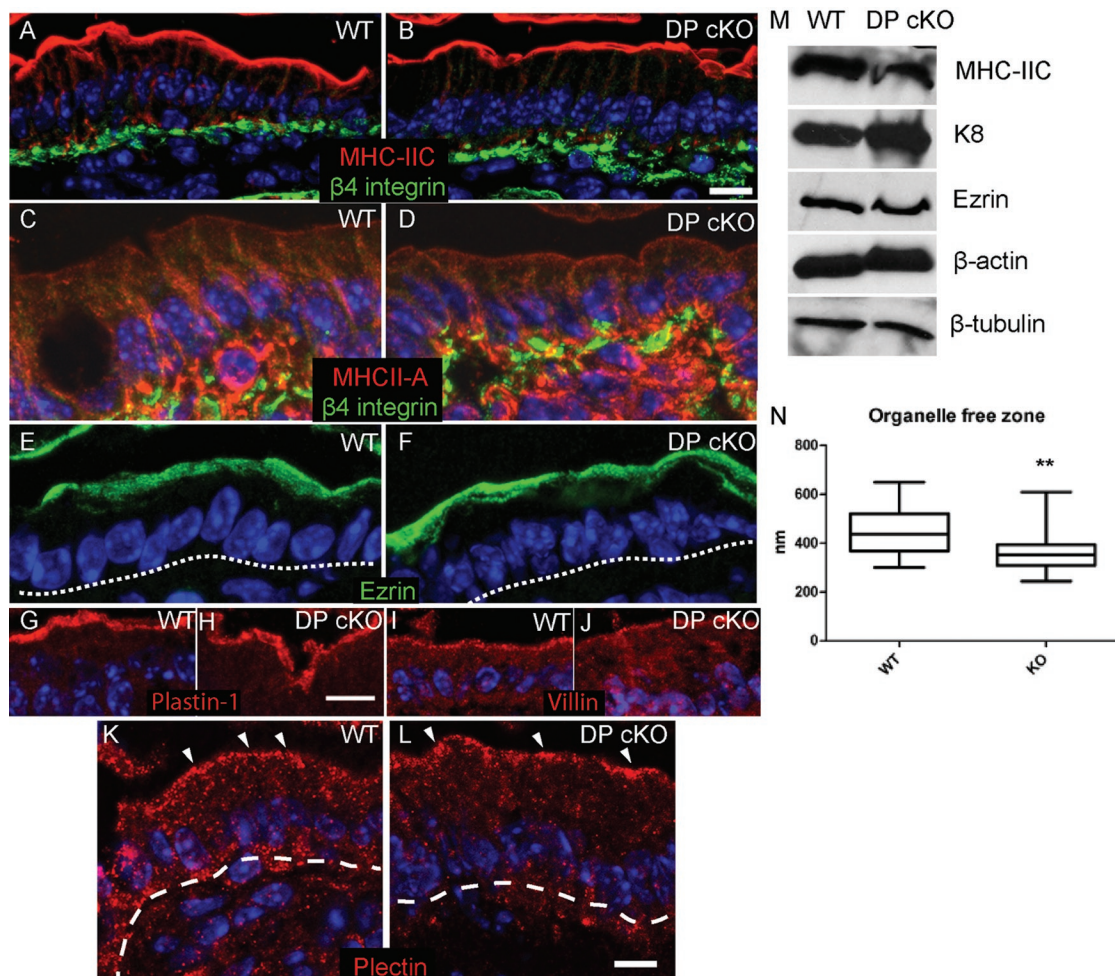


FIGURE 6: DP cKO intestine does not have terminal web defects. (A and B) Myosin heavy chain II-C (MHC-IIC; red) in WT and DP cKO villi. The basement membrane (green) is marked by $\beta 4$ -integrin. (C and D) Myosin heavy chain II-A (MHCII-A; red) in WT (C) and DP cKO (D) villi. The basement membrane (green) is marked by $\beta 4$ integrin. (E and F) Ezrin (green) localization in WT (E) and DP cKO (F) villi. The basement membrane is marked by a white dotted line. (A–F) Scale bar: 10 μ m, as shown in (B). All images are maximum-intensity projections. (G and H) Plastin-1 (red) in WT (G) and DP cKO (H) villi. Images are maximum-intensity projections. (I and J) Villin (red) in WT (I) and DP cKO (J) villi. (G, H, and J) Scale bar: 10 μ m, as shown in (H). (K and L) Plectin (red) in WT (K) and DP cKO (L) villi. Apical enrichment is highlighted by the white arrowheads. Basement membrane is marked by a white dotted line. Scale bar: 10 μ m. (M) Western blots of WT and DP cKO intestinal epithelial cells. (N) Quantification from transmission electron micrographs of the organelle-free zone in WT and DP cKO enterocytes. The edges of each box represent the 25th and 75th percentiles, and the whiskers are the minimum and the maximum. **, $p = 0.0003$.

mechanism(s) that organize microtubules. In differentiated enterocytes, centrosomal proteins, including the microtubule-nucleating protein γ -tubulin, become redistributed to the apical region of the cell (Meads and Schroer, 1995; Salas, 1999; unpublished data), in which they are thought to nucleate and anchor noncentrosomal microtubules. Furthermore, γ -tubulin is anchored to the apical region of the cell by intermediate filaments (Salas, 1999). Because the keratin filament network is not disrupted in DP cKO intestine, γ -tubulin maintains its proper localization, and can likely still organize microtubules. Alternatively, adherens junctions may also play a role in organizing microtubules. The protein Nezha can interact with microtubule minus ends and tether them to the zonula adherens in Caco-2 cells (Meng *et al.*, 2008). Whether Nezha is localized at the zonula adherens in the small intestinal epithelium, and whether it participates in microtubule organization in enterocytes, is unknown.

Unexpectedly, the loss of DP from the intestinal epithelium had a profound effect on microvillar architecture. The loss of DP resulted

in a reduction in microvilli length, with no apparent change in terminal web composition or organization of the actin filaments within the microvilli. One possible explanation for this effect is that the loss of DP, and therefore the loss of keratin filament attachment to the desmosome, results in a weakening of the terminal web. The terminal web is composed of several cytoskeletal components, all anchored at the apical junctional complex at the edges of the cell. This anchorage to the junctions allows the terminal web to deform and resist force without losing the tether to the membrane. The microvilli, which insert their actin rootlets into the terminal web, may exert a force on the terminal web as they grow out and protrude from the apical surface. The loss of the keratin filament anchorage to the desmosome may weaken the terminal web such that it cannot sustain long microvilli. Alternatively, it is possible that DP controls signaling to the actin cytoskeleton, resulting in changes in microvilli length.

The microvilli of DP cKO intestine look very similar to isolated brush borders that have been exposed to calcium. On addition of

calcium, isolated brush borders completely vesiculated their membrane overlying the microvilli, and the actin filaments of the microvilli disintegrated, while the rootlets remained unchanged (Burgess and Prum, 1982). This effect of calcium on microvilli has been attributed to the calcium-dependent, actin-severing activity of the actin bundler villin (Burgess and Prum, 1982; Ferrary *et al.*, 1999). DP has not been implicated in the regulation of calcium levels or villin activity, but whether it is directly involved in either of these processes requires further investigation.

Loss of myosin IA from the intestinal epithelium also results in short microvilli (Tyska *et al.*, 2005). However, myosin IA localizes specifically to the microvilli, in which it forms bridges between the actin filaments and the plasma membrane. We did not observe the large membrane herniations in the DP cKO intestine that were present in the myosin IA KO mice. Therefore the short microvilli phenotypes in each KO mouse are likely caused by different mechanisms.

In summary, DP is not an essential protein in the intestinal epithelium. While not required for tissue integrity or cell–cell adhesion, it is required for keratin filament anchorage to the desmosome. Furthermore, DP is essential for proper microvilli shape and length, suggesting cross-talk between the desmosome and the actin cytoskeleton.

MATERIALS AND METHODS

Immunofluorescence staining

For all analysis, the proximal small intestine was used. The most proximal 3–4 cm was removed from the mouse, flushed with cold phosphate-buffered saline (PBS), and cut open such that the epithelial layer was exposed. The intestine was mounted onto brown paper towel with the intestinal wall side down, and embedded in optimal cutting temperature compound orthogonal to the mold. Eight-micrometer-thick tissue sections were fixed in 4% paraformaldehyde (PFA) for 8 min at room temperature or in methanol for 2 min at -20°C or in acetone for 2 min at -20°C . For visualization of microtubules, the tissue was fixed in 80 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.9), 75 mM NaCl, 3 mM MgCl_2 , 1 mM CaCl_2 , 0.5% Triton X-100, 3% PFA, and 0.5% glutaraldehyde for 30 min at 37°C . After being washed in PBS, the tissue was treated with sodium borohydride for 15 min at room temperature. After washing overnight, the tissue was embedded in optimal cutting temperature compound. For Lis1 staining, tissue sections were fixed in acetone for 2 min at -20°C . All washing and blocking steps were performed in PBS with 1% Triton X-100. Primary antibody was incubated for 1 h. Primary antibodies used were mouse anti-DP I/II (Millipore, Billerica, MA), rat anti- $\beta 4$ integrin (BD Biosciences, Franklin Lakes, NJ), rabbit anti- γ -catenin (Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-E-cadherin (gift from C. Jamora, University of California, San Diego), rabbit anti-ZO1 (Invitrogen, Carlsbad, CA), rabbit anti-keratin-18 (gift from E. Fuchs, Rockefeller University, New York), mouse anti- β -tubulin (Sigma-Aldrich, St. Louis, MO), rabbit anti-ninein (gift from J. Rattner, University of Calgary), rabbit anti-Lis1 (Santa Cruz Biotechnology), rabbit anti-myosin heavy chain IIA (Covance, Princeton, NJ), rabbit anti-myosin heavy chain IIC (Covance), rabbit anti-plastin-1 (gift from F. Rivero, Hull York Medical School, United Kingdom), rabbit anti-villin (Cell Signaling Technology, Danvers, MA), rat anti-keratin 8 (TROMA-1; Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-keratin 17/19 (Cell Signaling Technology), and guinea pig anti-plakophilin 2 (Progen Biotechnik, Heidelberg, Germany).

Biotin barrier assay

The biotin barrier assay was adapted from previous reports (Furuse *et al.*, 2002). The most proximal 2 cm of the intestine was flushed with

cold PBS. Fifty microliters of a 1 mg/ml solution of sulfo-NHS-biotin (Sigma-Aldrich) was injected into the lumen of the intestine of WT and DP cKO intestine. For the positive control, a WT intestine was injected with 40 μl of a 1 mg/ml solution of sulfo-NHS-biotin in the presence of 2 mM EGTA. After 30 min, the intestines were flushed with cold PBS and processed for cryosectioning and staining with ZO-1 antibodies and streptavidin–fluorescein isothiocyanate (Invitrogen).

Transmission electron microscopy

Proximal small intestines from mice were isolated, opened longitudinally, and fixed in a solution of 2% glutaraldehyde, 4% PFA, 1 mM CaCl_2 , and 0.05 M cacodylate (pH 7.4), for 1 h at room temperature and then overnight at 4°C . Samples were washed in 0.1 M sodium cacodylate buffer containing 7.5% sucrose. Samples were postfixed in 1% osmium tetroxide in 0.15 M sodium cacodylate buffer for 1 h and then washed in two changes of 0.11 M veronal acetate buffer for 15 min each. Samples were placed into en bloc stain (0.5% uranyl acetate in veronal acetate buffer) for 1 h, washed in veronal acetate buffer, and then dehydrated in a series of 70, 95, and 100% ethanol. Finally, the samples were prepared for embedding in Epon resin. After sectioning, samples were imaged with a CM12 transmission electron microscope (Phillips, now part of FEI, Hillsboro, OR) run at 80 kV with an XR60 camera (Advanced Microscopy Techniques, Woburn, MA). Image acquisition was done using 2Vu software (Advanced Microscopy Techniques). Microvilli length and organelle-free zones were measured using the length tool in the AxioVision software (Zeiss, Jena, Germany) and analyzed in GraphPad Prism (La Jolla, CA). A Student's *t* test was used to determine statistical significance.

Scanning electron microscopy

Proximal small intestines from mice were isolated, opened longitudinally, and fixed in a solution of 2% glutaraldehyde, 4% PFA, 1 mM CaCl_2 , and 0.05 M cacodylate (pH 7.4), for 1 h at room temperature and then overnight at 4°C . Samples were washed twice with PBS for 10 min each. The PBS was removed, and 1% osmium tetroxide was added to the sample, which was incubated in the dark for 1 h. The samples were rinsed three times with PBS for 10 min each time. The samples were then dehydrated in a series of 30%, 50%, 70%, and 90% ethanol washes, each of which was repeated twice, and a 100% ethanol wash, which was repeated three times for 10 min each time. Ethanol was removed and tetramethylsilane was added for 10 min three times. The tetramethylsilane was removed, and samples were allowed to air-dry for at least 10 min. Samples were mounted with double-sided, 12-mm-width carbon tape (Electron Microscopy Sciences, Hatfield, PA) onto aluminum mounts with slotted heads and tapered pins (12.7-mm-diameter table, 3.1-mm-diameter pin, 7-mm height; Electron Microscopy Sciences). Samples were sputter-coated with gold using a Denton Desk IV vacuum sputter-coater set to 25% sputter set point and sputtered for 360 s. Samples were imaged on an FEI XL30 FEG-SEM (FEI, Hillsboro, OR) scanning electron microscope with the beam set to 10 kV. Scandium software was used to acquire images.

Western blotting

Intestinal epithelial whole-cell lysates were prepared by flushing the intestine with cold PBS, opening the intestine longitudinally, and scraping the epithelial cells with a square glass coverslip (#1.5, 22 mm \times 22 mm; VWR). Laemmli sample buffer was added to the cells, which were then boiled for 5 min. Samples were spun down, and the supernatant was removed and loaded onto SDS–PAGE gels.

ACKNOWLEDGMENTS

We thank Colin Jamora, Jerome Rattner, Francisco Rivero, Sylvie Robine, Elaine Fuchs, and Deb Gumucio for reagents; Julie Underwood for expert care of the mice; Phillip Cristopher and Michelle Gignac for assistance with electron microscopy; and members of the Lechler Lab for comments on the manuscript. This work was supported by grants from the March of Dimes, National Institutes of Health–National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01-AR055926), and Golfers Against Cancer.

REFERENCES

- Ameen NA, Figueroa Y, Salas PJ (2001). Anomalous apical plasma membrane phenotype in CK8-deficient mice indicates a novel role for intermediate filaments in the polarization of simple epithelia. *J Cell Sci* 114, 563–575.
- Bornslaeger EA, Corcoran CM, Stappenbeck TS, Green KJ (1996). Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell-cell interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly. *J Cell Biol* 134, 985–1001.
- Burgess DR, Prum BE (1982). Reevaluation of brush border motility: calcium induces core filament solution and microvillar vesiculation. *J Cell Biol* 94, 97–107.
- Ferrary E *et al.* (1999). In vivo, villin is required for Ca²⁺-dependent F-actin disruption in intestinal brush borders. *J Cell Biol* 146, 819–830.
- Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S (2002). Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 156, 1099–1111.
- Gallicano GI, Bauer C, Fuchs E (2001). Rescuing desmoplakin function in extra-embryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature. *Development* 128, 929–941.
- Gallicano GI, Kouklis P, Bauer C, Yin M, Vasioukhin V, Degenstein L, Fuchs E (1998). Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *J Cell Biol* 143, 2009–2022.
- Green KJ, Stappenbeck TS, Parry DA, Virata ML (1992). Structure of desmoplakin and its association with intermediate filaments. *J Dermatol* 19, 765–769.
- Grimm-Gunter EM, Revenu C, Ramos S, Hurbain I, Smyth N, Ferrary E, Louvard D, Robine S, Rivero F (2009). Plastin 1 binds to keratin and is required for terminal web assembly in the intestinal epithelium. *Mol Cell Biol* 29, 2549–2562.
- Jonkman MF, Pasmooij AM, Pasmans SG, van den Berg MP, Ter Horst HJ, Timmer A, Pas HH (2005). Loss of desmoplakin tail causes lethal acantholytic epidermolysis bullosa. *Am J Hum Genet* 77, 653–660.
- Karagiosis SA, Ready DF (2004). Moesin contributes an essential structural role in *Drosophila* photoreceptor morphogenesis. *Development* 131, 725–732.
- Lechler T, Fuchs E (2007). Desmoplakin: an unexpected regulator of microtubule organization in the epidermis. *J Cell Biol* 176, 147–154.
- Meads T, Schroer TA (1995). Polarity and nucleation of microtubules in polarized epithelial cells. *Cell Motil Cytoskeleton* 32, 273–288.
- Meng W, Mushika Y, Ichii T, Takeichi M (2008). Anchorage of microtubule minus ends to adherens junctions regulates epithelial cell-cell contacts. *Cell* 135, 948–959.
- Pinto D, Robine S, Jaisser F, El Marjou FE, Louvard D (1999). Regulatory sequences of the mouse villin gene that efficiently drive transgenic expression in immature and differentiated epithelial cells of small and large intestines. *J Biol Chem* 274, 6476–6482.
- Prahlad V, Yoon M, Moir RD, Vale RD, Goldman RD (1998). Rapid movements of vimentin on microtubule tracks: kinesin-dependent assembly of intermediate filament networks. *J Cell Biol* 143, 159–170.
- Rodriguez ML, Brignoni M, Salas PJ (1994). A specifically apical sub-membrane intermediate filament cytoskeleton in non-brush-border epithelial cells. *J Cell Sci* 107, 3145–3151.
- Salas PJ (1999). Insoluble γ -tubulin-containing structures are anchored to the apical network of intermediate filaments in polarized CACO-2 epithelial cells. *J Cell Biol* 146, 645–658.
- Salas PJ, Rodriguez ML, Viciano AL, Vega-Salas DE, Hauri H-P (1997). The apical submembrane cytoskeleton participates in the organization of the apical pole in epithelial cells. *J Cell Biol* 137, 359–375.
- Saotome I, Curto M, McClatchey AI (2004). Ezrin is essential for epithelial organization and villus morphogenesis in the developing intestine. *Dev Cell* 6, 855–864.
- Stappenbeck TS, Green KJ (1992). The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J Cell Biol* 116, 1197–1209.
- Sumigray KD, Chen H, Lechler T (2011). Lis1 is essential for cortical microtubule organization and desmosome stability in the epidermis. *J Cell Biol* 194, 631–642.
- Thomason HA, Scothern A, McHarg S, Garrod DR (2010). Desmosomes: adhesive strength and signalling in health and disease. *Biochem J* 429, 419–433.
- Tyska MJ, Mackey AT, Huang JD, Copeland NG, Jenkins NA, Mooseker MS (2005). Myosin-1a is critical for normal brush border structure and composition. *Mol Biol Cell* 16, 2443–2457.
- Vasioukhin V, Bowers E, Bauer C, Degenstein L, Fuchs E (2001). Desmoplakin is essential in epidermal sheet formation. *Nat Cell Biol* 3, 1076–1085.
- Womack WA, Barrowman JA, Graham WH, Benoit JN, Kvietys PR, Granger DN (1987). Quantitative assessment of villous motility. *Am J Physiol* 252, G250–G256.