Cell dynamics in fetal intestinal epithelium: implications for intestinal growth and morphogenesis

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SUMMARY

The cellular mechanisms that drive growth and remodeling of the early intestinal epithelium are poorly understood. Current dogma suggests that the murine fetal intestinal epithelium is stratified, that villi are formed by an epithelial remodeling process involving the de novo formation of apical surface at secondary lumina, and that radial intercalation of the stratified cells constitutes a major intestinal lengthening mechanism. Here, we investigate cell polarity, cell cycle dynamics and cell shape in the fetal murine intestine between E12.5 and E14.5. We show that, contrary to previous assumptions, this epithelium is pseudostratified. Furthermore, epithelial nuclei exhibit interkinetic nuclear migration, a process wherein nuclei move in concert with the cell cycle, from the basal side (where DNA is synthesized) to the apical surface (where mitosis takes place); such nuclear movements were previously misinterpreted as the radial intercalation of cells. We further demonstrate that growth of epithelial girth between E12.5 and E14.5 is driven by microtubule- and actinomyosin-dependent apicobasal elongation, rather than by progressive epithelial stratification as was previously thought. Finally, we show that the actin-binding protein Shroom3 is crucial for the maintenance of the single-layered pseudostratified epithelium. In mice lacking Shroom3, the epithelium is disorganized and temporarily stratified during villus emergence. These results favor an alternative model of intestinal morphogenesis in which the epithelium remains single layered and apicobasally polarized throughout early intestinal development.

KEY WORDS: Cell polarity, Shroom3, Apical constriction, Cell elongation, Interkinetic nuclear migration, Lumen formation, Mouse

INTRODUCTION

When formed at embryonic day (E) 9.5, the murine primitive gut tube possesses a central lumen surrounded by a polarized, endodermally derived epithelium (Wells and Melton, 1999; Zorn and Wells, 2009). Over the next 5 days, the epithelium progressively increases in girth (Mathan et al., 1976; Matsumoto et al., 2002). At E14.5, the thickened epithelium begins a drastic remodeling process whereby the previously flat luminal surface is converted into villi, which are finger-like epithelial projections with mesenchymal cores.

Careful histological studies of specimens from multiple species have provided an outline of morphological events involved in villus formation (Babyatsky and Podolsky, 2003; Dauca et al., 1990; Hashimoto et al., 1999; Madara et al., 1981; Mathan et al., 1976; Matsumoto et al., 2002; Toyota et al., 1989). In most of these investigations, the epithelium prior to E14.5 is characterized as multilayered (i.e. stratified) (Babyatsky and Podolsky, 2003; Dauca et al., 1990; Hashimoto et al., 1999; Madara et al., 1981; Mathan et al., 1976; Matsumoto et al., 2002; Toyota et al., 1989), although rare studies have indicated that it is single layered (i.e. pseudostratified) (Matsumoto et al., 2002). Several mechanistic studies of villus formation and intestinal growth have interpreted experimental data in the context of the stratified model (Cervantes et al., 2008; Kim et al., 2007; Saotome et al., 2004). However, no careful analysis of cell shape and cell dynamics in the intestinal epithelium has been carried out despite the fact that these factors have important implications for cell cycle compartmentalization, patterns of intestinal growth and mechanisms of epithelial remodeling.

Compartmentalization of mitosis differs in stratified and pseudostratified epithelia. For example, in the esophagus, which is a stratified epithelium of the gastrointestinal tract, the basal-most layer contains stem and transit-amplifying cells, which together provide the mitotic compartment. As cells leave the basal layer and move towards the luminal surface, they withdraw from the cell cycle and differentiate (for a review, see Croagh et al., 2008). In the transiently stratified epithelium of the branching mammary gland, mitotic cells are distributed throughout the multiple cell layers of the terminal end bud (Ewald et al., 2008). By contrast, in pseudostratified epithelia, such as the embryonic neural tube or developing retina, the cell cycle is uniquely tied to a process called interkinetic nuclear migration (INM) (Sauer, 1936). During INM, nuclei near the basement membrane synthesize DNA, then travel apically while in G2 phase, undergo mitosis at the apical surface and travel back to the basal surface in G1 phase. Thus, whereas mitotic figures are either basally located or distributed in stratified epithelia, mitosis is an apical event in pseudostratified epithelia. Owing to their constant apicobasal (AB) movement, nuclei appear staggered in a pseudostratified epithelium (Miyata, 2008). INM and, by implication, nuclear staggering, are a consequence of cell proliferation, as agents that inhibit proliferation interrupt INM (Baye and Link, 2008; Ueno et al., 2006).

The mechanisms that drive growth of intestinal girth are also predicted to differ in stratified versus pseudostratified epithelia. Stratified epithelia increase girth by adding cell layers, whereas pseudostratified epithelia thicken by progressive elongation of epithelial cells (Miyata, 2008). It has been shown that AB elongation of pseudostratified epithelial cells in the developing
neural tube depends on Shroom3, a PDZ binding domain-containing protein that localizes to the apical adherens junction (Haigo et al., 2003; Hildebrand, 2005; Hildebrand and Soriano, 1999). Interestingly, Shroom3 also controls apical constriction, another attribute shared by most pseudostratified epithelia (Haigo et al., 2003; Hildebrand, 2005; Lee et al., 2009; Lee et al., 2007; Nishimura and Takeichi, 2008; Taylor et al., 2008).

The exact nature of cell shape and cell polarity in the early intestine also impacts our interpretation of the mechanisms that drive the drastic remodeling of intestinal surface that occurs at E14.5. A stratified epithelium would remodel by cell reorganization and de novo polarization of central cells; indeed, existing models favor this strategy (see figure 7E in Saotome et al. (Saotome et al., 2004) and figure 1A in Kim et al. (Kim et al., 2007)). These models are supported by ultrastructural studies that link the initiation of epithelial remodeling to the de novo formation of apical surfaces at so-called secondary lumina located deep within stratified epithelial layers (Madara et al., 1981; Mathan et al., 1976; Toyota et al., 1989) and by recent studies in the zebrafish (Bagnat et al., 2007) and mouse (Saotome et al., 2004) intestine that have pinpointed some of the genes required for this process. Dynamic changes in cell polarity and the fusion of a network of secondary lumina in the context of a transient stratified epithelium were recently shown to drive the formation of murine pancreatic acini (Villasenor et al., 2010). However, in a pseudostratified epithelium, in which all cells already have an apical surface facing the lumen, a more likely mechanism driving remodeling would be cell shape change, coupled with expansion of the existing luminal surface.

Together, these findings highlight the importance of cell shape, cell polarity and cell cycle dynamics in understanding early intestinal epithelial morphogenesis. Here, we provide the first comprehensive analysis of cell dynamics in the early murine intestinal epithelium and establish a new model of intestinal morphogenesis. We show definitively that the early intestinal epithelium is pseudostratified, undergoes INM and grows in girth by progressive AB elongation that is dependent upon microtubules and actomyosin signaling. We find that villus formation does not occur by secondary lumen formation, but by expansion of the primary apical surface. Finally, we demonstrate that the actomyosin signaling protein Shroom3 is an important determinant of the single-layered pseudostratified epithelium. In the absence of Shroom3, apical surfaces are disorganized and the epithelium becomes temporarily stratified.

MATERIALS AND METHODS

Mice

Embryonic tissues were obtained from the following mouse lines: Mice that were hot start. mTmG, CaggCre and Shroom3 genotypes can be obtained from the Jackson Lab website.

Genotyping PCR

Genotyping PCR was performed with the GoTaq PCR kit (Promega M7122) and primers from Integrated DNA Technologies. All PCR cycles were hot start. mTmG, CaggCre and Shroom3 primer sequences and genotyping protocols can be obtained from the Jackson Lab website.

Tissue preparation

Tissues were fixed 1 hour to overnight in 4% paraformaldehyde and prepared for paraffin, OCT or agarose embedding. For paraffin, tissue was rinsed with PBS, dehydrated in ethanol and infused with paraffin. For OCT, tissue was washed with PBS, incubated overnight in 30% sucrose and embedded in OCT. Agarose samples were rinsed in PBS and embedded in 7% low melting point agarose. CaggmTmG samples were always agarose embedded. Paraffin blocks were sectioned at 5 μm, OCT blocks were sectioned at 8 μm, and agarose embedded tissue was vibratome sectioned at 150 μm. Sections were stained and imaged on an Olympus Fluoview 500 laser-scanning confocal microscope or Nikon eclipse E800 wide-field fluorescence microscope.

Immunostaining

Sections were deparaffinized in xylene, rehydrated, washed with PBS and then boiled in 10 mM sodium citrate (pH6) for antigen retrieval. Sections were incubated for 30 minutes in blocking buffer [1% BSA, 10% goat serum, 0.3% Triton X-100 in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl)]. Primary antibodies (details below) were diluted in blocking buffer and incubated overnight at 4°C. Molecular Probes Alexa Fluor 488-, 568- or 647-conjugated secondary antibodies (Invitrogen; 1:1000) with 10 μg/ml Hoechst (Invitrogen) were diluted in blocking buffer for 30 minutes at room temperature. Slides were mounted with ProLong Gold (Invitrogen).

Vibratome sections were permeabilized in 0.5% Triton X-100 in PBS, blocked (4% goat serum and 0.1% Tween 20 in PBS), incubated with primary antibody at 4°C overnight, incubated with Molecular Probes Alexa Fluor-conjugated secondary antibodies (Invitrogen; 1:1000) and 10 μg/ml Hoechst (Invitrogen) for 45 minutes at room temperature, and mounted with ProLong Gold between two glass coverslips. Images were obtained with an Olympus Fluoview 500 laser-scanning confocal microscope. z-stacks were 3D reconstructed using Imaris software (Bitplane).

X-Gal staining

Cryostat sections were air dried, washed in PBS and stained for 2 hours overnight in the dark at 37°C in X-Gal stain solution: 1 mM MgCl2, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 0.1 mg/ml ascorbic acid on a 4°C shaker. Tissues were washed with PBS and incubated in X-Gal stain solution for an additional 2 hours at room temperature. Sections were deparaffinized in xylene, rehydrated, washed with PBS and embedded. Paraffin blocks were sectioned at 5 μm, and agarose embedded tissue was vibratome sectioned at 150 μm. Sections were stained and imaged on an Olympus Fluoview 500 laser-scanning confocal microscope. z-stacks were 3D reconstructed using Imaris software (Bitplane).

Antibodies

β-actin-FITC (Sigma F3022; 1:1000), BrdU (Accurate Chemical and Scientific Corporation clone BU175/1; 2:100), BrdU (DSSHB clone G3G4; 1:400), β-catenin (β-cyt; BD Biosciences; 1:400), caspase 3 (Casp3; R&D AF835; 1:500), collagen IV (CollIV; Millipore A8765P; 1:500), crumbs 3 (1:5000), E-cadherin (Ecad; Invitrogen ECCD2 13-1900; 1:1000), Ecad (BD 610181; 1:1000), ezrin (Thermo Scientific MS-661-P1; 1:100; blocking from the M.O.M. kit (Vector Labs)), Gm130 (BD 610822; 1:250), Ki67 (Thermo Scientific rm-9106-S1; 1:400), pHH3 (Millipore 05-806; 1:200), PKC (Millipore 05-806; 1:200), phospho-histone H3 (pHH3; Millipore 6-570; 1:400), pHH3 (Millipore 05-806; 1:200), PKC (aPKC; Santa Cruz sc-216; 1:500), γ-tubulin (γ-Tub; Sigma T5326; 1:500; block over night), zona occludens 1 (ZO-1; Invitrogen 33-9100; 1:1000), Shroom2 [1:100 (Dietz et al., 2006)] and Shroom3 [1:100 (Hildebrand, 2005)].

BrdU labeling studies

Pregnant females were intraperitoneally injected with a pulse of 50 mg/kg BrdU and chased with 500 mg/kg thymidine. Tissues were paraffin embedded and stained with anti-BrdU. BrdU-positive nuclei were counted.

Organ culture

Fetal intestines were dissected in warm PBS and cultured in B27 medium (Gibco) with 1% Pen/Strep (Invitrogen) and 0.1 mg/ml ascorbic acid on Transwell plates (Costar 3428) at 37°C with 5% CO2. Intestines were cultured for 15 hours at the air-liquid interface in the presence or absence of mitomycin C (Mitoc; 5 μg/ml), blebbistatin (Blebb; 5 μM), or nocodazole (Nocod; 0.1-1.0 μM) prior to fixation, embedding, sectioning and immunostaining.

Electroporation

E13.5-14 BL6 intestines were dissected into ice cold PBS. Intestinal lumens were injected with 1 μg/μl pEGFP-C1 (Clontech) using a pulled glass needle and immediately electroporated with three pulses of 35 V (50 msec on, 500 msec off) using a low-voltage, square wave
Electroporator (Abud et al., 2004). Intestines were cultured for 24-48 hours on Transwell plates, agarose embedded, stained and imaged. DNA for electroporation was purified using the Qiagen EndoFree plasmid maxi kit.

**Scanning electron microscopy (SEM)**
Intestinal tissue samples were fixed in Bouin’s solution and dehydrated in ethanol followed by hexamethyldisilazane. Samples were critical point dried and mounted using double-sided tape. The intestinal surface was exposed by cracking with a sharp razor blade. Samples were then coated with gold/palladium under vacuum and imaged on an AMRAY 1910 field emission scanning electron microscope (FEG-SEM) using XStream imaging software.

**Statistical analysis**
Statistical analysis was performed using PASW 17.0.3 software (IBM). Each dataset was subjected to the Shapiro-Wilk test of normality and to Levene’s test for homogeneity of variances. All datasets met both criteria. Thus, one-way ANOVA with a Tukey post-hoc analysis was used to examine data in Figs 1, 4 and Fig. S1 in the supplementary material.

**RESULTS**

**Growth of the early intestinal epithelium**
To document growth in girth in the early intestinal epithelium we examined C57BL6/J (BL6) mice at E12.5, E14.5 and E16.5 (Fig. 1). Epithelial girth was recorded as distance from the basement membrane to the apical surface in intestinal cross-sections from the proximal duodenum, just distal to the pancreatic duct (Fig. 1), and from the distal ileum just proximal to the cecum (see Fig. S1 in the supplementary material). In both areas, growth in epithelial thickness (girth) between E12.5 and E14.5 is associated with an increase in the number of stacked nuclei. Girth drops precipitously upon remodeling, whereas cellular apical surfaces expand (Fig. 1B-D; see Fig. S1B-D in the supplementary material). The increase in nuclear layers between E12.5 and E14.5 could be due to increasing stratification of the intestinal epithelium or might reflect the progressive packing of nuclei in a pseudostratified epithelium. To further explore these alternatives, we investigated AB polarity and cell shape.

**Cell polarity in the early intestinal epithelium**
Markers that detect apical [atypical PKC (aPKC), ezrin (Ezr), β-actin, crumbs 3, ZO-1 (Tjp1 – Mouse Genome Informatics), collagen IV (CollIV)] and lateral [E-cadherin (Ecad); cadherin 1 – Mouse Genome Informatics] cell surfaces were examined by confocal imaging of vibratome-sectioned intestines. All five apical proteins are confined to the luminal surface of the epithelium at both E12.5 and E14.5 (Fig. 1B,C; Fig. 2A-E). Occasionally, small regions of apical staining are seen in deeper regions of the epithelium (e.g. Fig. 2D, arrowheads). However, when examined in the context of the entire z-stack, these regions are always contiguous with the central lumen. We found no evidence for isolated apical surfaces in deep regions of the epithelium. As expected, the basal marker CollIV was found exclusively at the circumference of the epithelial tube (Fig. 2C) and Ecad staining was located at the lateral surfaces (Fig. 2B,E). This distribution of polarity markers is consistent with a pseudostratified epithelium. However, a similar distribution of markers would be seen in stratified epithelia in which central cells lack both apical and basal connections.

**Fig. 1.** Growth in girth of the mouse fetal intestine and correlation with histological changes in the proximal duodenum. (A) Intestinal epithelial thickness at E12.5, 14.5 and 16.5 (n=6 for each stage). (B-D) Intestinal sections stained with Hoechst (blue) and for Ecad (green), aPKC (red) and CollIV (red) were used to measure girth (double-headed arrows). The apical surface expands from ~2 μm per cell to ~5 μm per cell (on the villus) between E14.5 and E16.5. A one-way ANOVA and Tukey HSD test revealed significant differences between the groups: F(2,20)=44; *, P<0.001. Error bars indicate s.d. Scale bars: 20 μm.

**Fig. 2.** Analysis of cell polarity in mouse E14.5 intestine. (A-F) The apical proteins ZO-1 (A), ezrin (B), aPKC (C), β-actin (D) and crumbs 3 (E) localize to the luminal surface. Occasional apical staining deeper within the epithelium (white arrowheads, D) is connected with the primary lumen in a different plane (see text). CollIV (C) stains the basement membrane surrounding the epithelial layer, and Ecad (B, E) outlines the lateral surface. Gm130 (F) stains Golgi that extend along the lateral surface. Apical and basal are marked by dotted yellow and white lines, respectively. Scale bars: 20 μm.
centrosome is positioned at the center of the apical surface; centrosome-to-centrosome distance reflects the apical cell size. As shown in Fig. 3A, centrosomes are densely packed at the luminal surface of BL6 epithelium, suggesting that cells are highly constricted.

Apical cell footprints were also examined in 100 µm vibratome slices of E14.5 duodenal tissue stained for ZO-1, a tight junction marker. Using 3D reconstruction software, a portion of the luminal surface was rebuilt from the confocal z-stack, revealing numerous small apical footprints (Fig. 3B). The few large footprints that were seen are mitotic cells, as discussed below. This ZO-1 pattern closely resembles that seen in the neural tube (Miyata, 2008), a known pseudostratified epithelium.

**Fig. 3. The intestinal epithelium is pseudostratified.**

(A) Centrosomes stained for γ-Tub concentrate at the apical surface. (B) A 3D reconstructed confocal z-stack from a vibratome-sectioned mouse intestine stained for ZO-1 (green) and with Hoechst (blue) reveals many small apical footprints (arrow) and fewer large mitotic apical footprints (arrowhead). (C-E) Reconstructed confocal z-stacks from vibratome-sectioned Cagg;mTmG fetal intestines show the shapes of individual EGFP-positive epithelial cells at E12.5 (C) and E14.5 (D, E). Movie 1 in the supplementary material is a video of E. (F) Analysis of 259 E14.5 EGFP-positive cells from Cagg;mTmG 3D reconstructions reveals that the epithelium is pseudostratified. Some cells at the edges of the z-stack could not be unequivocally analyzed (N/A). Scale bars: 20 µm.

Tracing cellular shape confirms that the intestinal epithelium is pseudostratified

We took several different approaches to visualize whole cells in the context of the intact epithelium. First, scanning electron microscopy (SEM) of E13.5 epithelium revealed epithelial cells stretching across the AB axis (see Fig. S2A in the supplementary material). Second, in cultured intestines 24 hours after electroporation of a luminally injected plasmid encoding CMV-EGFP, reconstructed confocal z-stacks from vibratome sections showed that cells expressing EGFP touched both the basal and apical surfaces (see Fig. S2B in the supplementary material); the nuclei of these cells were positioned basally in some cases and apically in others. Identical results were seen when E14.5 intestinal lumens from BL6 animals were injected with a fine micellar mixture of Dil-labeled oil in PBS and cultured on a Transwell membrane for 8 hours prior to confocal imaging (see Fig. S2C in the supplementary material).

These data are consistent with the idea that at least some cells of the early intestinal epithelium are pseudostratified. However, these techniques might favor the labeling of cells that touch the apical surface, leaving open the possibility that a population of basally oriented cells exists that do not stretch apically or that a population of central cells might exist without touching either the basal or apical surfaces. Thus, as a third approach, we utilized mTmG reporter mice (Muzumdar et al., 2007), which express myristilated tomato DsRed (Leimeister et al., 1999) in cell membranes in the absence of Cre recombinase and myristilated CMV-EGFP (Hepker et al., 1999) in cell membranes after Cre recombination. mTmG mice were mated with

![Image](https://example.com/image.png)

**Fig. 4. Intestinal nuclei undergo interkinetic nuclear migration (INM).** BrdU was used to track nuclear movement (see text). (A-C) Mouse intestinal sections stained with Hoechst (blue) and for BrdU (green), aPKC (red), CollIV (red) and pH3 (pink). After 40 minutes (A), BrdU is visible in S-phase nuclei along the basement membrane; mitotic (pH3-positive) nuclei are not BrdU positive (inset). After 120 (B) and 200 (C) minutes, BrdU-positive nuclei progressively migrate towards the apical surface. Colabeling of pH3 and BrdU is rare at 120 minutes and frequent at 200 minutes (insets). (D) Quantification of the proportion of non-basal BrdU-positive nuclei reveals progressive apical migration. A one-way ANOVA and Tukey HSD test revealed significant differences between the groups: F(2,9)=170, * P<0.001. Error bars indicate s.d.; n=4 intestines. (E) Localization of pH3-positive nuclei is apical. A one-way ANOVA and Tukey HSD test revealed significant differences between the groups: F(2,9)=352, * P<0.001. Error bars indicate s.d.; n=4 intestines. Scale bars: 20 µm.
CaggCreERT2 (Cagg (Hayashi and McMahon, 2002)) mice, and pregnant dams were gavaged with tamoxifen 24 hours prior to sacrifice. In Cagg;mTmG offspring, mT-labeled membranes of single cells were readily observed in the background of mT-labeled cells. A movie of a confocal z-stack (Fig. 3 and see Movie 1 in the supplementary material) reveals that individual cells are highly elongated and stretch across multiple sections within the z-stack, accounting for the fact that 2D images of histological sections rarely capture whole cells and instead give the false appearance of stratification. Reconstructions of multiple z-slices from E12.5 (Fig. 3C) and E14.5 (Fig. 3D,E) confirm that the majority of the cells stretch across the entire girth of the epithelium. Quantitation of 259 cells from the E14.5 z-stacks reveals that 83% of EGFP-positive cells stretch from the apical surface to the basement membrane (Fig. 3F). Another 4% touch only the apical surface; generally, these cells are rounded and might represent dividing cells. Finally, 13% of cells at the edges of the z-stack could not be unequivocally analyzed. We found no clear evidence for the presence of basally located cells that do not touch the apical surface and no evidence for centrally located cells that touch neither the basal nor the apical surface. Identical results were obtained in the distal ileum (see Fig. S1E-G in the supplementary material).

Together, the data from polarity studies and from several different labeling techniques are congruent and indicate that the early intestinal epithelium thickens between E12.5 and E14.5 by progressive cell elongation and progressive pseudostratification rather than by addition of cellular layers. Therefore, the murine intestinal tube is maintained throughout this period as a single layer of AB polarized epithelium.

Cell cycle dynamics in the early intestinal epithelium

Pseudostratified epithelia are typically highly proliferative and undergo a unique process known as INM, wherein nuclei move back and forth between the apical and basal surfaces in concert with the cell cycle (Miyata, 2008; Sauer, 1936; Ueno et al., 2006). To determine whether the early intestinal epithelium shares these characteristics, intestinal sections from E14.5 BL6 mice were stained for Ki67, a marker that is present on cells in all phases of the cell cycle except G0. The majority of epithelial cells are actively cycling (see Fig. S3A in the supplementary material). Casp3 staining revealed very little apoptosis (see Fig. S3B in the supplementary material).

To trace nuclear movements, pregnant dams were intraperitoneally (IP) injected at E14.5 with a pulse of the thymidine analog bromodeoxyuridine (BrdU); 20 minutes later, the same dams were IP injected with a thymidine chase to quench BrdU uptake. Nuclei in S phase incorporate BrdU and their links DNA in S phase, inhibiting cell cycle progression. After 15 hours, control intestines retained nuclear staggering, with three to four layers of nuclei visible (Fig. 5A). By contrast, intestinal cells in MitoC-treated intestines took on a more columnar shape without the S-G2-M phases of the cell cycle in the early intestinal epithelium. Finally, we found that mitosis takes place exclusively at the luminal (apical) surface (Fig. 4E), as it does in other pseudostratified epithelia (Miyata, 2008; Sauer, 1936; Ueno et al., 2006). Together, these data demonstrate that the pseudostratified intestinal epithelium undergoes INM.

Mechanisms controlling growth of epithelial girth in the early intestine

Cell elongation and INM occur concurrently during epithelial thickening, suggesting that INM movements and progressive staggering of cell nuclei could be responsible for the growth of epithelial girth. To test this, we interrupted the cell cycle in cultured intestines; previous studies have shown that cell cycle progression is required for INM (Ueno et al., 2006). E14 intestines were dissected from BL6 mice, placed into culture on Transwell membranes and treated with 5 μg/ml MitoC, an agent that cross-links DNA in S phase, inhibiting cell cycle progression. After 15 hours, control intestines retained nuclear staggering, with three to four layers of nuclei visible (Fig. 5A). By contrast, epithelial cells in MitoC-treated intestines took on a more columnar shape without
nuclear staggering (Fig. 5B). This change in cell shape did not affect epithelial thickness. Thus, cell cycle progression, nuclear movement and progressive nuclear staggering are not required for the maintenance of intestinal girth.

In other systems, both microtubule dynamics and actomyosin signaling have been implicated in the control of cell height (Levina et al., 2001; Picone et al., 2010). We tested whether either of these cytoskeletal signaling systems is essential for intestinal epithelial cell elongation. Intestinal organ cultures from Cagg;mTmG mice (E14) were treated with the myosin IIB inhibitor Blebb or the microtubule-polymerizing inhibitor Nocod. Blebb was used at 5 μM, a concentration previously shown not to affect the cell cycle (Schenk et al., 2009). After 15 hours of culture, control intestines retained their pseudostratified morphology, with three to four nuclear layers visible (Fig. 5C). However, Blebb-treated intestines exhibited drastically diminished cell height (Fig. 5D). Nuclear staggering was intact in Blebb-treated intestines, with two to three nuclear layers visible, and mitotic figures were found at the apical surface. These findings imply that, in the intestinal epithelium, cell elongation, but not INM, requires actomyosin signaling.

Cell height was also reduced in cultured E14 Cagg;mTmG intestines treated with 1 μM Nocod (Fig. 5E,F), implying a role for microtubules in cell lengthening. Since Nocod can also block cells in metaphase due to a failure of spindle formation, rounded cells at the luminal surface after 15 hours of culture confirmed the apical localization of mitosis. In Fig. 5E, one of the rounded mitotic cells expressing mG has a long process that connects to the basal surface, indicating that dividing cells normally maintain their basal contacts. Such basal processes have also been seen in neuroepithelium (Das et al., 2003; Miyata et al., 2001; Saito et al., 2003). We conclude that the increase in girth in the intestinal epithelium is due to the progressive lengthening of pseudostratified cells and that maintenance of epithelial cell height requires the microtubular network as well as actomyosin signaling.

**Shroom3 is required to maintain the proper apical organization of the intestinal epithelium**

Shroom3 is a PDZ-containing protein that localizes to the apical surface of pseudostratified epithelial cells, where it directs both apical constriction and AB elongation via the control of actomyosin signaling and γ-Tub organization (Hildebrand, 2005; Hildebrand and Soriano, 1999; Lee et al., 2007; Plageman et al., 2010). Recently, Shroom3 was shown to be important for the maintenance of proper epithelial cell shape in the *Xenopus* gut (Chung et al., 2010). In mouse, *Shroom3* is expressed in the E8.5 and E10.5 gut (Hildebrand and Soriano, 1999), but expression at later stages has not been examined and the role of Shroom3 in mouse intestinal development is unknown.

We examined intestinal *Shroom3* expression between E13.5 and E15.5 using the gene-trap mouse line *Shroom3Gt/Gt*, in which a lacZ reporter gene is spliced into the *Shroom3* transcript, allowing detection of *Shroom3* expression via X-Gal staining (Hildebrand and Soriano, 1999). In this model, *Shroom3* activity is greater in the proximal intestine than in the distal intestine at all times examined. Expression peaks at E14.5 (see Fig. S4A,B in the supplementary material) and is reduced with the emergence of villi at E15.5 (see Fig. S4C,D in the supplementary material). Antibody staining confirms that both Shroom2 and Shroom3 are expressed at E14 and localized to apical junctions (see Fig. S4E,F in the supplementary material).

To investigate the role of Shroom3 during intestinal morphogenesis, we analyzed Hematoxylin and Eosin-stained intestinal sections from wild-type (WT) and *Shroom3* null (*Shroom3Gt/Gt*) mice harvested between E14 and E17 (Fig. 6A-J). Prior to E14.5, *Shroom3* null intestine appears similar to that of wild type. However, at E14.5, significant collections of rounded, sometimes pyknotic, cells are observed at the central lumen (Fig. 6G); this phenotype is dramatic in the proximal intestine and more subtle distally. Casp3 staining confirms that some of these rounded central cells are apoptotic (see Fig. S3C in the supplementary material). At E15, layers of ectopic cells are adherent to emerging villi, and at E16, clusters of ectopic cells are observed at the tips of lengthening villi (Fig. 6H,I). By E17, villi appear normal (Fig. 6J); we posit that cell turnover on the villi eventually clears the phenotype.

We next examined the polarity of the rounded ectopic cells in *Shroom3* null intestines. Analysis of apical markers (αPKC, γ-Tub and phalloidin staining) reveals that apical surfaces are greatly expanded in this model (Fig. 7A-J). The lateral marker Ecad emphasizes the rounded geometry of these cells, as compared with the tall, thin and apically constricted WT cells (Fig. 7E,F). Ectopic cells appear tightly adhered to their neighbors. Since Shroom3 localizes to apical junctions (see Fig. S4E,F in the supplementary material) and organizes the apical actin network in neural epithelium and MDCK cells (Hildebrand, 2005; Hildebrand and Soriano, 1999), we examined apical actin distribution using the F-actin-binding toxin phalloidin. This
Supplementary material. Scale bars: 20 μm in A,B,E-J; 5 μm in C,D.

**Fig. 7.** Expanded apical surfaces in Shroom3 null intestines. (A,B) In Shroom3-deficient mouse proximal intestines, rounded cells fill the lumen; some are pyknotic. aPKC-stained surfaces are expanded (red). (C,D) The expanded apical surface is emphasized by the greater distance between γ-Tub-stained centrosomes (green) in Shroom3 null intestines as compared with WT controls. γ-Tub images are rebuilt in Helicon Focus from three consecutive images over 5 μm. (E,F) In WT embryos, epithelial cells are elongated and apically constricted. In the absence of Shroom3, central cells are rounded and some luminal surfaces show Ecad staining. Cells appear to be tightly adhered to one another. (G,H) Strong phalloidin (green) staining detects an expanded F-actin network at the apical surface of Shroom3 null intestines. (I,J) Three-dimensional surface renderings of confocal z-stacks from phalloidin staining. A sheet-like apical actin network covers emerging villi in WT intestines; this network is disorganized and highly branched in Shroom3 null intestines. I and J rotate in Movies 2 and 3 in the supplementary material. Scale bars: 20 μm in A,B,E-J; 5 μm in C,D.

Analysis reveals an expanded and ramified F-actin network in Shroom3 null mice (Fig. 7G,H). Reconstructed surface renderings of phalloidin staining (Fig. 7I,J and see Movies 2 and 3 in the supplementary material) demonstrate that, in control mice, the apical actin network from contiguous cells forms a smooth sheet over emerging villi (Fig. 7I). By contrast, the apical actin network of Shroom3-deficient intestines appears rough and highly disorganized (Fig. 7J), which is likely to be due to the presence of numerous ectopic cells with expanded apical surfaces.

**Shroom3 plays a crucial role in the maintenance of a single-layered pseudostratified epithelium**

To unravel the underlying cellular etiology of the Shroom3 mutant phenotype, we first examined pHH3 staining within the central ectopic cell population. None of the central cells exhibits pHH3 staining, confirming that these are not collections of rounded mitotic cells. Notably, pHH3-positive cells are not located at the central lumen, as in WT mice, but are positioned beneath the ectopic cell populations (Fig. 8A,B, arrows). However, because the apical surfaces of ectopic cells are greatly expanded, these mitotic cells are still in contact with apical surfaces as in WT mice (Fig. 8A,B). Thus, we posited that the rounded cells might be postmitotic cells that, in the absence of Shroom3, failed to reassume a pseudostratified shape after cell division and became trapped luminally.

To test this idea directly, we traced the origins of the central ectopic cells using a BrdU pulse-chase strategy (Fig. 8C-E). Intestines from E14.5 WT and Shroom3 null mice were harvested at 40, 120 and 200 minutes after a single BrdU pulse and thymidine chase. Analysis of BrdU-labeled nuclear position with time revealed that Shroom3 mutants undergo INM with kinetics that are similar to WT mice, including colabeling of pHH3 and BrdU at 120 and 200 minutes after BrdU injection (Fig. 8D,E, arrowheads). No BrdU labeling is detectable within the central rounded cells 40 minutes after BrdU administration (Fig. 8C). However, rare BrdU-positive cells are detectable in this population at 120 minutes and increased numbers of central nuclei are labeled 200 minutes after BrdU administration (Fig. 8D,E). BrdU-labeled nuclei arrive in this central position 120-200 minutes after the BrdU pulse, which is also the time required to complete the S-G2-M phase of the cell cycle. Together, these findings indicate a critical requirement for Shroom3 as cells complete mitosis and shift from their rounded postmitotic condition to re-establish an elongated and apically constricted shape. Cells that fail to reassume their pseudostratified shape might lose their attachment to the basal surface and undergo anoikis, or might remain attached to neighboring cells and survive as a temporarily stratified population, until those anchoring cells are shed during the process of normal cell turnover. Thus, Shroom3 signaling is required to maintain the single-layered, pseudostratified epithelium and to properly shape the smooth apical surface of emerging villi.

**DISCUSSION**

Numerous studies in rat, mouse, sheep, pig and rabbit have characterized the intestinal epithelium as stratified prior to villus remodeling (Cervantes et al., 2008; de Santa Barbara et al., 2003; Dekaney et al., 1997; Dekaney et al., 2007; Hashimoto et al., 1999; Kim et al., 2007; Madara et al., 1981; Mathan et al., 1976; Saotome et al., 2004; Sbarbati, 1982; Toofanian and Targowski, 1982; Toyota et al., 1989). In all of these studies, the histological and microscopic techniques used were unable to capture the entirety of the extremely long, thin epithelial cells that stretch from the basal to apical surfaces. Through genetic labeling techniques and 3D reconstruction, we demonstrate that the early mouse intestinal epithelium is in fact pseudostratified and relies, in part, on Shroom3 to maintain orderly pseudostratification. This revised
model of intestinal organization, in which the intestinal tube maintains its AB polarity and its pseudostratified shape during early growth and during the initiation of villus morphogenesis, has substantial implications for the interpretation of previous and future functional studies of the developing intestine.

Our findings challenge the notion that secondary lumen formation is a central driver of intestinal remodeling, as previously suggested by early morphological studies (Mathan et al., 1976) and by the finding of malformed and fused villi in Ezr null mice (Saotome et al., 2004). Our 3D reconstructions indicate that, although villus development indeed entails an increase in apical surface, this does not derive from secondary lumen formation as previously thought. Rather, all expansion is at the apical surface itself. Thus, these data have novel implications for intestinal remodeling and suggest that cell shape plays a crucial role in villus development.

An appreciation that the early murine intestinal epithelium is pseudostratified also demands that current models of intestinal lengthening be re-examined. On the basis of the phenotypic comparison of WT and Wnt5a null mice, Cervantes et al. posited that the intestine grows in length via radial intercalation of stratified epithelial cells (Cervantes et al., 2008). Specifically, BrdU labeling patterns in WT E11.5 intestines led to the proposal that cells delaminate from the basal lamina, forming a temporarily stratified epithelium. Delaminated cells were posited to divide and then re-intercalate into the basal epithelial layer, expanding it lengthwise. In Wnt5a null mice, BrdU-labeled cells accumulate at the luminal surface. Hence, intestines exhibit increased girth, but severely reduced length, suggesting a defect in radial intercalation movements. Mice lacking Ror2, a receptor that binds Wnt5a, also exhibit a thickened epithelium and, again, altered radial intercalation has been proposed as the mediator of this phenotype (Yamada et al., 2010). From our current study, it is now clear that the authors were observing INM, but interpreting nuclear movements as cellular movements in the context of an incorrect stratified epithelium model. Indeed, radial intercalation is not possible in the context of a pseudostratified epithelium because the cells are already in a single layer.

In this regard, it is interesting that many pseudostratified epithelia (e.g. the developing neural tube, retina and, as demonstrated here, the intestinal epithelium) are rapidly proliferating tissues. It has been speculated that, in the neural tube, pseudostratification and INM are adaptations that allow increasingly dense packing of the actively proliferating progenitors needed for expanding the number of differentiated neurons and, thus, the size of the brain (Miyata, 2008; Smart, 1972). In the intestine, pseudostratification (and INM) might similarly allow the tight packing of a bolus of tall, thin progenitor cells, which, when reshaped to columnar during epithelial remodeling, would quickly and efficiently extend intestinal length. It will be important to test whether factors that interfere with INM also interfere with progenitor expansion and intestinal lengthening. Indeed, we are currently investigating the kinetics of the cell cycle and the efficiency of INM in the Wnt5a model.

The mechanisms that control the growth of intestinal girth have not been previously identified. Our studies reveal that progressive elongation of pseudostratified cells drives this aspect of epithelial growth. Further, the elongated shape of these pseudostratified cells requires an intact microtubule network as well as actomyosin signaling. Interestingly, both of these cytoskeletal systems have been shown to be regulated by members of the Shroom family in other pseudostratified epithelia (Dietz et al., 2006; Haigo et al., 2003; Hildebrand, 2005; Hildebrand and Soriano, 1999; Lee et al., 2009; Lee et al., 2007; Plageman et al., 2010). We provide the first detailed study of the crucial role of murine Shroom3 in intestinal cell shape. We show that in the absence of Shroom3, a subset of postmitotic cells is unable to reestablish the pseudostratified shape and becomes trapped luminally, creating a temporarily stratified epithelium. These ectopic cells are rounded, with a greatly expanded apical surface area. Rounded central cells have also been seen in the Xenopus intestine after transfection of dominant-negative Shroom3 (Chung et al., 2010), suggesting that Shroom3 plays an evolutionarily conserved role in intestinal epithelial morphology.

In Shroom3 null intestines, some cells retained their normal elongated shape. Compensation by intestinally expressed Shroom2 could potentially explain this conundrum, as the cytoskeletal signaling properties of Shroom2 and Shroom3 are similar (Lee et al., 2009). Overall, our data indicate that Shroom3 is primarily responsible for two key events at the apical surface: the establishment of the pseudostratified cell shape (with apical constriction) following mitosis, and the organization of the apical
actin network in contiguous cells at the surfaces of emerging villi. These activities might be connected. Indeed, we noticed that although Shroom3 null intestinal epithelia were generally disorganized, the collections of rounded ectopic cells occasionally took the shape of rudimentary villi (e.g. Fig. 6G). It is possible that the downregulation of Shroom3 expression that we noted at E15.5 is necessary for the expansion of apical surface detected during villus formation in WT mice (Fig. 1). An important future goal is to test whether overexpression of Shroom3 prevents or delays villus formation.

The studies described here represent the first comprehensive analysis of cell shape, cell cycle dynamics and cell polarity in the early intestinal epithelium. All data are congruent with the conclusions that the epithelium is pseudostratified, that increasing cell height is responsible for epithelial thickening, and that Shroom3 drives apical surface dynamics and modulates cell shape in the developing intestine. Given the established role for Shroom proteins in the cell shape and remodeling of many epithelia, it is important to identify which upstream factors control Shroom3 (and Shroom2) expression levels in the developing gut. Recent studies indicate that Pax6 is upstream of Shroom3 in the developing mouse lens placode (Plageman et al., 2010), and Ptx3 transcription factors have been identified as regulators of Shroom3 in Xenopus gut and neural tube (Chung et al., 2010). Ptx1 and Ptx2, as well as Pax6 (weak) and Pax7, are expressed in the E14.5 mouse intestinal epithelium (www.genepaint.org). It will be important to determine whether any of these transcription factors are involved in remodeling the E14.5 intestine through dynamic regulation of Shroom signaling.

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Figure S2
Figure S4