Chapter IV
Discussion

In this thesis, we examined the dramatic morphological changes in the small intestinal epithelium during the development of the first villi. The villi are integral for maximizing the surface area for nutrient absorption, and are required for proper growth and homeostasis. However, the initial events involved in rapidly “carving out” the villi from the thick pseudostratified embryonic intestinal epithelium had not been carefully studied. For decades, villi were believed to form from a stratified epithelium via formation and fusion of de novo secondary lumens and concurrent intercalation of the stratified cells to form a single layer (Mathan et al., 1976). Previously, the phenotype of the Ezrin null mouse, with ectopic lumens that appear to be residual secondary lumens, was thought to support this hypothesis (Saotome et al., 2004). However, in recent studies we demonstrated that the epithelium is pseudostratified, not stratified, and secondary lumens never normally form in the developing intestine (Grosse et al., 2011); in such a setting, intercalation cannot occur. Interestingly, our studies now show that while the wild type epithelium is always a single-layer and devoid of secondary lumens, the loss of EZRIN causes stratification as well as formation of ectopic secondary lumens.
The work described in Chapter II demonstrates that villus domains are initially established by patterned epithelial invaginations in the proximal intestine at E14.5. We find that these invaginations are associated with dividing cells. This was a novel observation that compelled us to compare the developing intestine to other systems in which cell division and lumen extension are related. In the *Drosophila* tracheal placode, patterned invaginations are the result of a two-step process. In the first step, circumferential forces passively constrict a group of cells; in the second, mitosis facilitates invagination (Kondo and Hayashi, 2013). In the intestine, these epithelial folds are preceded by the formation of mesenchymal clusters directly beneath the epithelium, which form such that each cluster defines one villus domain (Karlsson et al., 2000; Kolterud et al., 2009; Walton et al., 2012; Walton et al., 2016). Clusters location is patterned by mesenchymal Bmp signaling such that they form in a Turing field-like manner (Walton et al., 2016). Thus, we reasoned that this cluster pattern might somehow be transmitted to the epithelium to generate a patterned field of apical invaginations.

We find that signals from these clusters cause the adjacent epithelial cells to shorten in the apical-basal dimension and expand laterally. These shape changes enact a compressive force on epithelial cells between clusters, which may provide a mechanism for transmitting this patterning information from the mesenchyme to epithelium to demarcate villi. We utilized *in silico* modeling to confirm that these cell shape changes can generate a passive pressure on the inter-cluster epithelium. When a cell divides within this constricted region, invagination occurs. Together, this work suggests a previously undocumented role for intraepithelial physical forces in morphogenesis of the murine small intestine. Expansion of *in silico* simulations will inform further refinements to the *in vivo* model.
The phenotype of mice lacking the apical protein EZRIN (fused villi and ectopic lumens) (Figure III-1) was studied in Chapter III. Especially of interest was the mechanism of formation of ectopic lumens, as they normally do not develop in the intestine. Because other studies both in vitro and in vivo suggest that EZRIN maintains proper spindle angle (Casaletto et al., 2011; Hebert et al., 2012), the role of the mitotic spindle in intestinal morphogenesis was a major focus of this analysis. In the early small intestinal epithelium, like other pseudostratified epithelia that undergo interkinetic nuclear migration (IKNM), this angle is tightly controlled such that both daughter cells maintain an apical and basal process after cell division. Without EZRIN, cell division angle is randomized relative to the lumenal surface and the epithelium becomes stratified (Figure III-3). Treatment of explanted intestines with blebbistatin, a Myosin II inhibitor known to cause misorientation of cell divisions in the zebrafish embryo (Campinho et al., 2013), recapitulates the altered spindle angle and ectopic lumen phenotype present after EZRIN loss, reinforcing the importance of spindle angle control in intestinal morphogenesis.

It is clear that loss of Ezrin before villus formation (Chapter III and Saotome et al., 2004) and in the adult after crypts form (Casaletto et al., 2011) results in fused villi. In Chapter III, deletion of Ezrin during the first week of life, between the formation of villi and crypts, also resulted in a fused villus phenotype. This indicates that these fusions always independent of the timing of EZRIN protein removal. Moreover, lineage tracing studies revealed that a relevant contribution to the phenotype likely stems from the inability of cells to rearrange in relation to their neighbors. This may in turn be due to previously noted changes in junctional stability after EZRIN loss (Casaletto et al., 2011).

In addition to advancing our understanding of villus morphogenesis, this thesis also raises several interesting questions for further study. Below, I examine some of these questions and
propose future experiments for their exploration based on preliminary data and evidence from the existing literature.

**The role of forces on and within the epithelium in villus formation**

Several sources of force act on the early intestinal epithelium, both originating external to and within the epithelium. Throughout villus morphogenesis, the expanding pseudostratified epithelium is circumferentially constrained by inner circular muscle. Just before villi form, this highly proliferative epithelium results in a very tightly packed structure with tall, narrow cells. When mesenchymal clusters begin to form at E14.5, they induce shape changes in the overlying epithelial cells; this exerts patterned intraepithelial forces on the inter-cluster epithelium. Within these regions under high tension, a dividing cell initiates a rapid invagination event accompanied by a T-shaped fold. As described in Chapter II, patterning of clusters, epithelial shape change, and villus demarcation take place on an extremely rapid timescale; we estimate that a new villus domain is defined within minutes.

This novel perspective on the factors involved with intestinal morphogenesis prompts many questions for further study, including: a) What signals from clusters promote the initial epithelial cell shape change? b) Can alterations in cluster patterning induce a change to invagination pattern? c) Though the *in silico* model suggests that mechanical forces are sufficient to initially pattern folds, is there a role for soluble signaling molecules in this process? d) Is mitosis in the inter-cluster epithelium specifically patterned by surrounding forces? e) At the resolution of mitosis, how do the daughter cells separate? f) Is there a role for peristalsis, a coordinated muscular contraction, in villus formation?
**Factors causing cluster-mediated epithelial cell shortening**

Mesenchymal clusters are robust signaling hubs, expressing components of many pathways, including Hh, Bmp, PDGF, and Wnt (Karlsson et al., 2000; Walton et al., 2016; Walton et al., 2012). However, the signal or series of signals that is responsible for shortening the adjacent epithelial cells has yet to be determined.

One pathway that has been relatively unexplored in the small intestine has been the TGFβ pathway. There is evidence for TGFBRII expression in both the intestine epithelium and mesenchyme (Ng et al., 2002) (Figure IV-1A) and the ligands are mesenchymally expressed, with initial evidence that one of them (TGFB3) is restricted to clusters (Figure IV-1F-H). However, not much is known about the role of TGFβ signaling in the small intestine. In other systems, such as the *Drosophila* ovarian epithelium, this pathway causes cell shape to change from a cuboidal to squamous structure via cytoskeletal rearrangements (Brigaud et al., 2015).

Preliminary functional evidence for TGFβ signaling mediating cell shape change in the small intestine comes from experiments determining the connection between TGFβ signaling and the transcription factor ELF3. Constitutive *Elf3* deletion prevents the transition from pseudostratified to columnar epithelium, perturbs villus architecture, and decreases epithelial TGFBRII expression (Ng et al., 2002). These phenotypes are rescued when TGFBRII is overexpressed in the epithelium (Figure IV-1B-E) (Flentjar et al., 2007).

Determining whether TGFβ signaling mediates epithelial shape change over clusters can be done using inhibition or over-activation of TGFβ signaling with pharmacologic and genetic models. The small molecule SB431542 specifically inhibits TGFBRI (Inman et al., 2002); treatment in *ex vivo* culture may mediate global reduction of TGFβ signaling. Additionally, some genetic models are available for gain or loss-of-function analysis. The genetic overexpression
model of TGFBRII described above can be further used to study the effect of increased TGFβ signaling (Flentjar et al., 2007) and a recently developed floxed allele of Tgfb2 can be used to specifically delete this ligand from mesenchymal clusters (Ishtiaq Ahmed et al., 2014) by combining it with the Gli1CreERT2 allele, active in Hh-responsive cluster cells (Walton et al., 2016). Complications may arise from the potential redundancy of multiple TGFβ ligands and receptors. Additionally, although the expression of Tgfb3 appears to be the most relevant for development of clusters and villi, a floxed allele is currently unavailable.

This analysis may provide insight into the connection between epithelial cell shape changes and intraepithelial forces required for folding. If modulations in TGFβ signaling change cell shape without affecting cluster patterning, this will inhibit the transfer of cluster pattern to the overlying epithelium, and will likely cause the pattern of folds to be disconnected from cluster pattern. This would further emphasize the integration of intraepithelial forces and signaling in villus demarcation.

**Exploring the relationship between cluster and invagination patterning**

The model proposed in Chapter II indicates that the patterning of mesenchymal clusters is transmitted through mediating shape changes in the overlying epithelium that exert forces on the intervening epithelium. When cells undergo mitotic cell rounding in these regions, a patterned series of invaginations forms *in vivo* and *in silico*. To enhance understanding of the role of external forces to patterning villus demarcation, changing the overall constraint on the intestine or the patterning or size of mesenchymal clusters can be done.

The intestinal explant culture system is a very powerful system for modulating the development of the small intestine; previously, this system has allowed visualization of villus
development for up to three days ex vivo (Chapter II, III, and Grosse et al., 2011; Walton et al., 2012; Walton et al., 2016). Harvesting the intestines and culturing ex vivo will likely cause relaxation of circumferential pressure from the muscle and serosal layers of the intestine. Some evidence for this comes from reduced epithelial height and density that is typically seen in cultured intestines. Cutting the intestinal tube open and culturing it as a two-dimensional sheet can further reduce circumferential constraint. Because cluster pattern is driven by morphogen gradients (Walton et al., 2016), these manipulations are expected to reduce intraepithelial forces without affecting mesenchymal cluster patterning. These phenotypes can be assessed using both scanning electron microscopy (SEM) and cross sections. SEM offers a three-dimensional view of the apical surface and allows the number and pattern of folds to be compared. With reduced external pressure, we predict that folds will be shallower and potentially incomplete between early villi. However, because signals from clusters cause shortening of overlying cells as well as withdrawal of those cells from the cell cycle, these processes will continue to occur in culture. Therefore, villi will eventually emerge, though they will have less clearly demarcated boundaries. In cross sections, although clusters will develop normally, fewer T-shaped folds will be present. This highlights an important distinction: although folds develop in vivo as a feature of the pressures impinging on the developing intestine and provide a mechanism for the epithelium to rapidly generate villus boundaries, these folds may not be essential for villus formation in explant cultures.

Artificially increasing constraint on the intestine is another way to investigate the effects of these forces. To accomplish this, silk tubes of different sizes can be fabricated into which the early intestine can be threaded, similar to studies performed in the chick (Shyer et al., 2013). As the intestine grows in girth, these non-elastic tubes will constrain radial growth. Alternatively,
intestines can be grown in micro-patterned wells of different widths; intestines in narrower wells will be under increased constraint. Such manipulations may increase fold frequency or promote epithelial folding before clusters are present. It will be of interest to determine whether such folds are associated with mitotic cells.

One limitation of these experiments is that all of these manipulations not only affect the epithelium, but also modulate forces in the surrounding tissue layers. Thus, even if increased constraint produces extra convolutions, these may not truly be “villi”, but simply buckling of the highly proliferative epithelium into non-physiologic structures. Such a phenomenon has been described using in silico models of epithelial tissue sheets; compression above a certain threshold will cause convolutions (Hannezo et al., 2014). If this result is observed, distinguishing “true” villi from non-physiologic convolutions must involve consideration of how they are patterned and if they are associated with clusters. Expansion of the model developed in Chapter II to represent the intestine as a 3D cylinder may allow insight as to how forces are distributed within the in vivo developing intestine and the effects of these experiments.

To more specifically examine the effects of forces on the epithelium, the pattern and size of the mesenchymal clusters can be changed. This approach will directly test the connection between shape changes in cells over the mesenchymal clusters, generation of intraepithelial forces, and fold generation. Investigators in our laboratory have studied the effects on villus morphogenesis of several different small molecules that modulate cluster size and pattern (Walton et al., 2016; Walton et al., 2012). To increase cluster size, intestines can be treated with Dorsomorphin, an inhibitor of Bmp signaling. At very large doses, Dorsomorphin treatment merges clusters into stripes (Walton et al., 2016). These larger clusters further shorten the overlying epithelium and exert a higher force on inter-cluster regions. Because we find that this
intraepithelial force contributes to fold formation, this treatment may result in folds that form earlier and/or are deeper. If mesenchymal clusters merge at high doses, the force distribution within the epithelium will be altered, potentially patterning folds into “stripe-like” structures. Conversely, treatment with cyclopamine, an inhibitor of Hedgehog (Hh) signaling, decreases cluster size (Walton et al., 2012). This treatment, in contrast to Dorsomorphin treatment, will likely reduce the number of apical invaginations.

Modulation of Hh or Bmp signaling in this manner could, in principle also affect the epithelium, complicating this analysis. However, we know that while the epithelium expresses Bmp receptors, genetic deletion of epithelial Bmp receptors has no effect on initial villus morphogenesis (Walton et al., 2016). Also, because Hh receptors and downstream transcription factors are not expressed in the epithelium (Kolterud et al., 2009; Walton et al., 2012), modulation of that pathway cannot directly affect the epithelium. Of course, indirect effects on epithelial cell behavior could result from modulation of other signaling pathways in the mesenchyme that act independently on the epithelium. Thus, for these experiments it will be important to titrate the dose of the inhibitor in these experiments and utilize multiple mechanisms to inhibit the same pathway (for example, to inhibit Hh signal, utilize both cyclopamine and the blocking antibody, 5E1).

Another serious limitation of these experiments is their reliance on the explant culture systems, which, as stated above, might globally relieve tissue forces independent of small molecule treatment. Our preliminary studies of Dorsomorphin treatment during villus formation show that initial apical deformations form, but fewer sharp folds are visible between villus domains (Figure IV-2). To circumvent this issue, genetic strategies can be employed. For example, a genetic knockout of Bmp signaling can be performed to specifically affect cluster
size without culturing the intestine. By crossing the $Alk3^{\text{flox}}$ with $Gli1^{\text{CreERT2}}$ alleles, Bmp signal reception is specifically removed from mesenchymal clusters, phenocopying the effect of Dorsomorphin treatment (Walton et al., 2016). Analysis of the apical surface in these mice will reveal how altering cluster size affects folding frequency and depth in vivo. Furthermore, comparison with Dorsomorphin-treated intestines may provide deeper insights into the effect of the explant culture system.

Although technically challenging, live imaging of the evolving surface of the developing intestine during cluster formation would provide insight into the dynamics of cell shape changes. To properly visualize these cells, a combination of the $\text{Pdgfr}^{\alpha\text{EGFP}}$ allele, which allows for visualization of mesenchymal clusters (Walton et al., 2012), $\text{Shh}^{\text{Cre-eGFP}}$, and $\text{ROSA}^{{\text{mTmG}}}$, to trace the membranes of epithelial cells (Muzumdar et al., 2007), will be required. Confocal imaging of the apical portion of these intestines in regions overlying clusters may show the lateral expansion of these cells that have been observed in cross section, similar to the analysis conducted in Chapter II (Figure II-4). Furthermore, analysis of these shape changes after application of small molecules to alter cluster size and patterning can show the effect clusters have on the epithelium, reinforcing the connection between mesenchymal and epithelial patterning.

*Molecular signaling in the context of fold formation*

Although we hypothesize that cluster-mediated forces within the developing intestinal epithelium could directly pattern fold formation, these forces are likely enhanced or reinforced by differential gene expression. Mesenchymal clusters are signaling centers for a wide array of pathways (Kolterud et al., 2009; Walton et al., 2012); some of these signaling molecules clearly change cell shape and others likely change cell cycle dynamics. We know that the epithelium
over the clusters rapidly responds to these signals. For example, even as initial villi are emerging, cells above clusters turn off SHH and PDGFA and begin to withdraw from the cell cycle (Kolterud et al., 2009). As a result, proliferation becomes restricted to the intervillus zone. A careful analysis of the timing of gene expression changes in the epithelium with respect to the initial formation of mesenchymal clusters and epithelial folds has not yet been performed, and represents an important next step.

Though it is clear that clusters affect the overlying epithelium, it is also possible that cluster-produced signaling molecules could diffuse from the clusters and act on the inter-cluster epithelium. For example, this region begins to express Axin2, a canonical Wnt target gene, shortly after villus emergence (Figure IV-3A-C). Because Wnt signaling is not active before E14.0 in the proximal duodenum, this is likely to represent an initial localized activation of the pathway (Li et al., 2009). At this point, it is unclear what these activation signals are and where they originate. It seems unlikely that clusters could be the source of Wnt ligands that act on distant intervillus regions, because these ligands typically bind to extracellular matrix molecules and therefore may not travel far from their source (Maurice and Korswagen, 2014). Though the source of Wnt activation is an interesting and still unsolved problem, it leads to a broader question: what mechanisms activate and maintain the compartmentalization of proliferative and non-proliferative epithelial compartments as villi emerge?

One method to begin to address local changes in gene expression during villus morphogenesis would be to conduct a targeted transcriptome analysis during the first round of cluster formation using laser capture microarray (LCM). Using this approach, clustered and unclustered mesenchyme, along with epithelium adjacent to clusters and inter-cluster epithelium could be sampled. Such an analysis might reveal newly activated signals, but validation will be
required to address localization of the signaling proteins and receptors, allowing the
directionality of the signaling events to be assessed.

Equally interesting is the question of whether the force exerted on the inter-cluster
epithelium directly activates epithelial gene expression. Possible evidence for force-induced gene
expression is our recent and unpublished finding of the differential expression of CD44v6 in the
epithelium after clusters have formed by before villi have emerged. CD44v6, a hyaluronan
receptor that has the potential to be activated by tissue tension (Chopra et al., 2014), is expressed
only in the inter-cluster epithelium and the highly apically constricted cells on the edges of the
early intestinal epithelium (Figure IV-3D-E). These are the two regions where we predict higher
tissue tension due to compression. Interestingly, in addition to the role of Cd44v6 in mechano-
transduction, it also is a downstream target of Wnt signaling.

To further explore this early sign of intervillus compartmentalization, it will be important
to first construct a clear timeline of CD44v6 localization during villus demarcation: preliminary
studies indicate that it is not expressed at E13.5 (Dr. Jason Spence, personal communication). If
confirmed, this will indicate that this represents new activation of this gene or protein. The
expression of CD44v6 in the presence of Wnt inhibitors can be studied in intestinal explant
culture to determine whether its expression is in response to Wnt activation or tissue forces.
Additional studies in which small molecules or genetic strategies are used to alter epithelial
forces, as outlined above, could further define the connection between mechanical force and
CD44v6 expression.
Determining whether intraepithelial forces pattern mitosis

Another important feature of the model presented in Chapter II is the direct connection between fold formation and mitosis. Amazingly, 40% of all dividing cells are found at the tip of an apical fold, a remarkable finding considering that these regions constitute a very small portion of the overall intestinal surface area. Mitosis is important for both fold formation and, likely, acceleration of this process, but why do certain cell divisions cause folds when others do not? Two distinct mechanisms could account for this association: a) divisions may occur at stochastic locations throughout the early epithelium but folds may happen only when cells enter mitosis in areas subject to the correct pattern of forces; or b) cells might be specifically induced to divide as a result of the surrounding tension that also creates a fold. Studies using in vitro systems and the Drosophila wing disc offer support to both of these hypotheses: cells under increased tension are more likely to proliferate, a process dependent on Hippo signaling (Nelson et al., 2005; Rauskolb et al., 2014), and proliferative cells can alter the cell biology of mitosis, such as their spindle angle, based on external tissue forces (Mao et al., 2013).

The first membrane invaginations in the intestinal epithelium appear over a timeframe of a few minutes, and occur within a background of continuously proliferating epithelial cells. Epithelial cells over clusters have received signals to withdraw from the cell cycle, and presumably the cells between clusters do not receive a similar signal. However, if in addition to suppressing cell division in the adjacent epithelium, whether the clusters actively promote proliferation in the inter-cluster epithelium due to the pattern of forces they exert on the surrounding tissue has not been fully explored. This could be assessed by harvesting the intestine at E14.5, when cluster formation initiates proximally (Walton et al., 2012), and opening the tube longitudinally to view the apical surface as a 2D sheet. Visualizing clusters with PDGFRα and
mitotic cells with pH3 will allow determination of the relationship between the predicted intraepithelial forces exerted by clusters and the pattern of mitotic cells. A short pulse of nocodazole (less than 6 hours) could allow for simultaneous visualization of an increased number of mitotic cells if the amount in fixed tissue is too small to determine a pattern. However, care will need to be taken such that the intraepithelial forces are not altered by a long period of culturing \textit{ex vivo}.

Additionally, the connection between intraepithelial forces and mitotic pattern could be further examined by changing the pattern of clusters using small molecule treatments noted above and observing effect on the distribution of mitotic cells. If increasing cluster size increases intraepithelial pressure, we predict the number and distribution of mitotic events will be altered, supporting mechanism (b) above. If there is no connection between intraepithelial forces and cell divisions, the pattern of mitosis should be not depend on the cluster pattern. In this case, the forces may simply pattern regions that will invaginate when the next mitotic event occurs, as in mechanism (a).

\textit{Resolving daughter cell segregation at invaginations}

Although we have shown an association between dividing cells and apical fold extension, the fate of daughter cells after the division completes is unknown. We have found some pairs of recently divided cells that have apical surface along the cytokinetic plane (Figure IV-4A-B), suggesting that at least some of these cells may be deposited onto opposite sides of the newly formed fold, that is, onto separate villi.

One way to analyze how often these divisions result in segregation of daughter cells onto separate villi is to use lineage tracing. In preliminary studies, we employed the Confetti system
(ROSA<sup>Confetti/+</sup>), which relies on induced Cre activity to activate one of four fluorescent proteins (nGFP, RFP, mCFP, and YFP) (Snippert et al., 2010). Using the Shh<sup>CreER</sup> driver, we induced with tamoxifen at E13.5 and harvested embryos at E14.5 or E15.5 (24 and 48 hour induction) (Figure IV-4C). Clone cell number increased over time in a manner consistent with the rapid rate of epithelial proliferation previously determined (Figure IV-4D) (Grosse et al., 2011). The spatial distribution of clones containing two or more cells was then analyzed. Interestingly, only about 20% are segregated between multiple villus domains at both times tested (Figure IV-4E). Figure IV-4F and 4G shows two 3D reconstructions of 100 µm vibratome sections of the intestine after 48 hours of recombination to highlight examples of clones being distributed to one (Figure IV-4F) or two villi (Figure IV-4G). These preliminary data suggest that the proportion of clones that are split between villus domains is relatively low. In light of the observation that 40% of dividing cells at E14.5 are associated with invaginations, this suggests that only a subset of daughter cells of these divisions populate multiple villi. It also suggests that boundaries between villi are set relatively early in their development. These interesting data need to be further supported with future experiments.

A related question is: even if only a few cells are delivered to two different villi, how is cytokinesis resolved in those cell divisions such that the two daughter cells are anchored correctly within the epithelium? Members of our laboratory have been using high-resolution cell shape tracing to follow dividing cells in the pre-villus epithelium throughout mitosis and cytokinesis. This technique makes use of the ROSA<sup>m<sub>mTmG</sub></sup> reporter allele, which labels membranes (Muzumdar et al., 2007), along with an inducible Cre driver and tamoxifen delivered at low levels to activate the mGFP reporter at a low frequency. These studies reveal that cytokinesis begins at the basal process such that it is divided first. However, during the progression of
cytokinesis, one of the two basal processes regresses such that by the end of cytokinesis, only one of the two daughter cells remains attached to the basal lamina. The other cell must then reattach to the basement membrane (Dr. Sha Wang, unpublished).

The fate of the basal process in cells dividing at invaginations has not yet been determined. However, it is clear that cytokinesis proceeds differently in these cells compared to cells dividing elsewhere, because the type of cell division that does not form folds results in an apically attached, bent midbody (Figure IV-4H). In contrasts, cells dividing at invaginations have straight midbodies (Figure IV-4I). Furthermore, the apical surface is either not indented at all or slightly indented into a V-shape for most dividing cells, while cells dividing at invaginations are characterized by a very small apical surface, connected to the lumenal surface by a T-shaped structure. High-resolution cell shape tracing of the dividing cells at membrane folds will yield new insight into the fate of the basal process during those divisions. It is possible that this division also involves the loss of only one cell’s basal process. Alternatively, both basal processes may regress and reattach to their respective new villus domains. Ideally, performing this shape tracing on live intestinal tissue would also resolve the fate of each daughter cell as such divisions proceed.

*Role of active contractile forces in villus formation*

Another characteristic of the adult intestine that is essential for its function is peristalsis. This coordinated contraction of muscles moves intestinal contents from the mouth to the anus, facilitation digestion and absorption of nutrients (Burns et al., 2009). This occurs due to action of the inner circular and outer longitudinal smooth muscle layers that surround the whole intestinal
tube and is coordinated by the enteric nervous system (ENS) (Burns et al., 2009; Roberts et al., 2010).

Contractions of the intestinal tube are first observed in the duodenum beginning around E13.5; these are more consistent at E14.5 (Roberts et al., 2010). This timing is especially interesting, as villus development also initiates at this time. Because our data suggest that intraepithelial forces assist in patterning and formation of apical folds, active tissue-wide contractile events may also affect the efficiency of villus demarcation.

To assess the effect of these contractions on villus development, the ex vivo culture system can be used in concert with small molecules to block early peristalsis. Interestingly, at this time, such contractions do not require nerve activity, as they are unaffected by culturing with tetrodotoxin and still occur in a genetic mouse model that lacks an ENS. Instead, these early contractions are myogenic and inhibited by treatment with cobalt chloride, which inhibits Ca^{2+} entry into muscle fibers (Roberts et al., 2010). Therefore, the effect of cobalt chloride treatment during villus morphogenesis on both the ability to block early peristaltic contractions and the resulting fold formation efficiency could be assessed. Tetrodotoxin, which is not expected to have any effect on these contractions, could be used as a treatment control.

**Lessons from the Ezrin null mouse model: Fused villi and formation of ectopic lumens**

In addition to shape changes affecting the epithelium, our model for villus morphogenesis takes into account the strength of the apical actin web at the apical surface and force transmission within the inter-cluster epithelium. The apical actin web includes actin-rich microvilli, which may contribute to apical strength, as well as cell-cell junctions to maintain epithelial integrity,
which is important for epithelial cell rearrangement and force transduction. Both of these cellular components are affected by Ezrin loss.

_Determining the connection between fusions and altered apical stiffness_

It is widely known that Ezrin cross-links the apical surface to the actin cytoskeleton (Fehon et al., 2010), suggesting it has the capability to modulate cytoskeletal strength. _In vitro_ studies on MDCK cell monolayers have shown that increasing the number of contacts between EZRIN and PIP2, an apical phosphoinositide, increases apical membrane tension (Braunger et al., 2014), while loss of Ezrin reduces membrane tension (Brückner et al., 2015). In the Ezrin null intestine, TEM analysis shows a disorganized apical actin web, frequent blebs (Casaletto et al., 2011), and disrupted apical brush border (Saotome et al., 2004). These findings suggest that the apical surface is less stiff without EZRIN, which may lead to ineffective villus carving observed by SEM (Figure III-1). Initial work using our mechanical model developed in Chapter II suggests that decreased apical tension can decrease folding efficiency, although this effect is highly variable and depends on the amount by which the tension is reduced.

A surprising and still unexplained aspect of the analysis of the Ezrin null phenotype is that in the adult intestine, the apical brush border exhibits increased Rho activity and more proteins at the junctional complex, suggesting these junctions are more rigid (Casaletto et al., 2011). Overall, those data predict that the adult Ezrin null intestinal surface may be stiffer than the wild type intestine. This discrepancy might be due to differing dynamics of force in the adult and fetal epithelium, or might reflect the fact that measurements of membrane stiffness in cell monolayers _in vitro_ do not accurately predict the situation _in vivo._
To begin to resolve these questions, the stiffness of the apical surface in vivo can be measured by atomic force microscopy (AFM) in the fetal intestine as had previously been done in MDCK cells. This procedure uses a small cantilever probe to deform the apical surface, measuring the force required for a specified displacement. This generates a force vs. displacement curve, which can be used to calculate Young’s modulus, a measure of the substrate’s strength (Brückner et al., 2015). Performing this analysis on E14.0 intestines that have been longitudinally opened to allow access of the probe to the flat apical surface will determine the difference in apical strength between wild type and Ezrin null intestines. If stiffness is reduced in the absence of EZRIN, this will corroborate the in vitro studies as well inform parameters for further computational modeling. It will then be of interest to determine whether the adult intestinal epithelium similarly has a reduced stiffness, to explore possible age-dependent changes in this parameter. If stiffness is increased at this time, the connection between this trait and phenotype can be confirmed using pharmacological treatments; Y-27632, a ROCK inhibitor (Matthews et al., 2006), can reduce Rho activity in the setting of EZRIN loss, which may correct the folding defects.

If the embryonic intestine has a higher apical stiffness, additional pharmacological studies can further elucidate the connection between the actin network strength and villus morphogenesis. For example, Latrunculin A (inhibits actin nucleation) and Smifh2 (inhibits Formin-mediated actin assembly (Rizvi et al., 2009)) treatments should reduce apical tension, which, if this is the cause of the Ezrin null phenotype, may rescue the villus formation defect.
Alterations to junctional stability affect villus morphogenesis and homeostasis

With the work presented in this thesis, it is now clear that Ezrin loss at any point during life, from embryonic until adult stages, causes fused villi; these structures are indistinguishable from each other. Additionally, the phenotype, regardless of the age of initiation, progressively worsens over time, suggesting that a continually occurring process creates these fusions. These two attributes suggest that fusions at all ages have a common underlying cause.

Cell-cell junctions have been hypothesized to be important in the development of the intestine (Saotome et al., 2004). Further insights into their importance in villus morphogenesis can be accomplished three approaches: studying the distribution of cell clones in the Ezrin null embryo, analyzing the effects of aPKC-pseudosubstrate (aPKC-PS) treatment, and studying the Crb3 knockout mouse model.

First, studying how dividing epithelial cells distribute in the pre-villus epithelium without Ezrin can further inform our understanding of cell dynamics in the neonatal intestine. Recently, investigators in our lab showed that dividing cells disperse along the long axis of the intestine and rarely remain continuous (Dr. Sha Wang, unpublished). Clonal analysis can be conducted in the Ezrin null pre-villus epithelium by combining the Ezrec allele described in Chapter III with ShhCreER and ROSAmTmG, followed by induction with tamoxifen at E13.5 and collection at E14.5. If loss of EZRIN contributes to increased junctional strength, as predicted by other studies (Chapter III and Casaletto et al., 2011), these clones will likely be more contiguous along the proximal-distal axis of the intestine.

Second, as discussed in Chapter I, PRKCZ (aPKC) is important for the establishment of apical cell polarity by forming a complex with PAR-6 and CDC42 adjacent to the tight junction (Chen and Zhang, 2013; Karner et al., 2006). Both in vitro and in vivo aPKC mutations result in
impaired polarity and disrupted junctions (Suzuki et al., 2001), and aPKC inhibition weakens junctions (Nunbhakdi-Craig et al., 2002). With this in mind, we treated developing wild type and Ezrin null intestines with aPKC-pseudosubstrate (aPKC-PS), which specifically inhibits aPKC kinase activity. In wild type intestines, this treatment enhanced fold formation in the inter-cluster epithelium even in the absence of compression by the cells adjacent to mesenchymal clusters (Figure IV-5A-B). However, Ezrin null intestines were found to be resistant to this effect (Figure IV-5C-D). One potential explanation is that proper junctional strength is critical for correct formation of invaginations. aPKC-PS may cause ectopic folds by reducing the strength of cell-cell junctions, while the stronger junctions in Ezrin null intestines may explain their resistance to aPKC-PS. The effect of aPKC-PS treatment on junctional integrity needs to be further assessed by immunofluorescence and TEM.

Third, the Crb3 null intestine was recently demonstrated to develop fused villi (Whiteman et al., 2014). CRB3 is a transmembrane protein that defines apical surface, assists in assembly of tight junctions (Tepass, 2012), and interacts with EZRIN via its intra-cellular FERM-binding domain (Bulgakova and Knust, 2009; Whiteman et al., 2014). These direct and indirect connections to both cell-cell junctions and the cytoskeleton allow CRB3 to generate and transmit intracellular forces (Datta et al., 2011). Though intestines deficient in CRB3 do not show significantly altered TJ structure by TEM, this phenotype has only been analyzed in late embryonic development (E18.5) and has not been studied biochemically (Whiteman et al., 2014).

Further analysis of junctional stability in this mouse model using lineage tracing and biochemical strategies could reveal the extent to which the phenotypes of Crb3 and Ezrin mutations stem from a common defect in junctional complexes. Additionally, studying the Crb3 model at early time points will determine if villus fusions develop concurrently with
morphogenesis. Interestingly, although CRB3 and EZRIN are known to interact, loss of either protein does not affect the localization of the other (Chapter III and Whiteman et al., 2014), suggesting a parallel, not linear, pathway for how these perturbations generate fused villi. Combining these knockout alleles into a single mouse model may yield further insight into these mechanisms; if CRB3 and EZRIN loss cause fused villi by affecting junctions in different ways, loss of both is likely to cause an even worse phenotype than loss of either protein alone. A double-heterozygous $Crb3^{+/-};Ez^{+/-}$ mouse model may also yield a phenotype.

Previous studies of the Ezrin null mouse model in the adult demonstrate that cell-cell junctions are abnormally lengthened and additional proteins are present in these structures. This led to the conclusion that these junctions are more stable, and it was proposed that this leads to the fused villus phenotype at this time (Casaletto et al., 2011). The first functional results of this effect are presented in Chapter III, wherein lineage tracing with the Confetti system indicates that cells do not exchange neighbors as readily in the absence of EZRIN (Figure III-7).

The dynamics of epithelial cell movement through the epithelium both before and after crypt formation suggests a mechanism by which impaired junctional rearrangement leads to fused villi. In the adult intestine, which has established crypts, cells must dramatically rearrange their junctions to exchange neighbors at two locations: as they emerge from the crypt onto the crypt shoulder and as they exit the crypt shoulder and travel up the villus. In the neonatal intestine, which lacks crypts, cells must undergo this rearrangement as they move from the inter villus to the villus domain. Our studies on the neonatal intestine reveal that fusions begin basally and travel up the villi with time (Figure III-6), indicating that the epithelial shape change at the base of the villus is a critical process; this likely also plays a role in developing fusions in the adult intestine.
Overall, cell-cell junctions appear to be an important structure in both the development and maintenance of villus architecture in the intestine. Further studies in which forming and modifying junctions is impaired may yield a unified model of how these fusions form at all ages.

Investigating the mechanisms underlying ectopic lumen formation

It is interesting that although ectopic lumens have been previously observed in the embryonic intestine (Saotome et al., 2004), the mechanism of their formation has not been defined. We have determined for the first time that the Ezrin null epithelium is stratified (Chapter III), differentiating it from the wild type intestine. Thus, ectopic lumen formation may utilize a mechanism that is not seen in normal development. Broadly, these lumens could be formed de novo by polarization of a stratified epithelium at already established cell boundaries (Hick et al., 2013; Villasenor et al., 2010) or by trafficking of apical components to the cytokinetic plane during cell division as occurs in the zebrafish neural keel or in MDCK cysts suspended in extracellular matrix (Buckley et al., 2013; Jaffe et al., 2008; Schlüter et al., 2009; Taniguchi et al., 2015; Tawk et al., 2007) (Figure IV-6).

Formation of internal lumens in the context of a stratified epithelium is best exemplified by the development of pancreatic acini (Villasenor et al., 2010). In that system, transient stratification of the epithelium is followed by development of cellular rosettes and subsequent formation of microlumens at the rosette center. These events appear to be independent of cell division, instead relying on cell shape changes and alterations in cell polarity (Villasenor et al., 2010). Likewise, in the developing thyroid follicle, punctate intracellular regions, marked with EZRIN, are initially observed. These vesicles traffic to the center of cellular rosettes, fuse, and enlarge. Lumen expansion depends on VEGFA signaling; without it, apical domains are defined,
but these regions fail to expand. This can be rescued by ectopic addition of VEGFA or co-culture with endothelial cells (Hick et al., 2013).

Another type of lumen forming process requires cell division. The morphological aspects of lumen formation in this process are similar in the neural keel and in MDCK, Caco2, and hESC cells. In the neural keel, a central lumen is generated in the solid epithelium by means of a specialized cell division called a crossing (c-) division. In this division, when a cell from one side of the neural keel undergoes mitosis, apical components (such as Pard3) become concentrated at the cytokinetic plane. One daughter cell crosses over the tissue midline, such that after division resolves, the two daughter cells are segregated onto opposite sides of the newly formed lumen. Blocking cell division lowers the efficiency of lumen formation (Buckley et al., 2013; Tawk et al., 2007). Similarly, individually plated MDCK cells traffic CRB3 along the mitotic spindle during division, forming an apical membrane initiation site (AMIS) at the cytokinetic plane (Schlüter et al., 2009). Other cell types also form lumens during their first cell divisions, although the mechanisms differ slightly (Jaffe et al., 2008; Taniguchi et al., 2015); specifically, hESC may form an intracellular organelle, termed the “proto-lumen”, which is trafficked intact to the cytokinetic plane during division (Dr. Kenichiro Taniguchi, unpublished). Overall, the frequency that a cell division-dependent mechanism forms lumens in cultured cells is remarkable.

Recently, this process was also shown to generate bile canalicular lumens in vitro and in vivo (Wang et al., 2014). In these studies, apical components traffic to the midbody in a Par3-dependent manner during cell division, very similar to the zebrafish c-division. Preliminary in vivo analysis confirms that the midbody is closely associated with developing bile canaliculi and that tight junctions form around the midbody before the patent lumen is established. Thus, this
process uses the midbody as a landmark for the forming bile canaliculus. During expansion of the
Canalicular lumen, cell division orients such that the midbody is adjacent to the established
Lumen. Thus both daughter cells remain in contact with this luminal surface (Wang et al., 2014),
similar to how cell division is controlled in the pre-villus intestinal epithelium.

There are two ways for this type of lumen-forming cell division to result in disconnected
Lumens in the Ezrin null model. First, a cell may begin to divide at the apical surface, but the
division plane may be oriented obliquely such that the cleavage plane of the daughters does not
Communicate with the apical surface. Such a division process could create an ectopic lumen
Between the two cells that is disconnected from the main lumen (Figure IV-6B). Alternatively,
Following an oblique cell division of this type, one cell will remain apical while the second will
Lose its apical contact. If this second cell then enters mitosis in a position distinct from the apical
Surface and then undergoes a lumen-forming division, this will create a lumen within the
Epithelium (Figure IV-6C). In both of these cases, cell division must occur by a different
Mechanism than in the wild type intestine, because we have shown that under normal
circumstances, intestinal epithelial cells do not undergo lumen-forming cell division.

In Chapter III, we characterized an oblique spindle angle in many dividing cells of the
Ezrin null epithelium (Figure III-4) and we showed that his epithelium is stratified (Figure III-3).
However, our preliminary evidence suggests that vast majority of cell divisions occur at apical
Surfaces, even in the absence of EZRIN. We have not detected clear evidence of intraepithelial
cell divisions, though this needs more careful examination. Additionally, we failed to detect
Intracellular apical protein trafficking. This could be due to the combined fact that mitosis itself
Is very fast, less than one hour, and these lumen-forming division events may be rare. The use of
The nocodazole block described in Chapter III may increase the number of cells undergoing
division in any one section, and can help to confirm where cells divide in the Ezrin null epithelium.

To further evaluate the mechanism of lumen formation in mice deficient in Ezrin, it will be important to examine additional markers of apical trafficking to sites disconnected from the primary apical surface. Apical components such as PAR3, CDC42, and Rab11 endosomes should be tracked. Blocking cell division with aphidicolin, a DNA polymerase inhibitor (Spadari et al., 1982), will not affect cellular trafficking, allowing us to examine whether cell divisions themselves are required for lumen formation. Because the thyroid model of lumen expansion after coalescence depends on VEGF signaling (Hick et al., 2013), culturing Ezrin null intestines with VEGFA inhibitors will assess whether decreased ectopic lumen expansion is seen. Additionally, it would be of interest to study the wild type intestine treated with blebbistatin to determine the localization of EZRIN and other apical proteins along with the association of mitotic cell with ectopic lumens.

Finally, if these studies fail to show evidence of a cell division-mediated mode of ectopic lumen formation, it will be important to consider the microlumen strategy exhibited by pancreas and thyroid epithelial cells, both of which are also endoderm-derived (Spence et al., 2011). This process can be best followed by examining ZO-1 expression during the formation of cellular rosettes. In support of this mechanism, it is intriguing that many of the small ectopic lumens in the Ezrin null model appear to have a rosette-like structure.

**Final Thoughts**

In this work, our studies of villus morphogenesis in the wild type mouse has established how the thick pseudostratified intestinal epithelium is rapidly carved into patterned villus
domains to increase the overall surface area of the developing intestine. These studies revealed a previously unknown connection between classical cell-cell communication by soluble signaling molecules and tissue mechanics. First, soluble signals pattern mesenchymal cluster location via a self-organizing Turing field (Walton et al., 2016). These clusters signal to the overlying epithelial cells to alter their cell shape and proliferation dynamics; this altered epithelial cell shape exerts a patterned field of mechanical forces on the inter-cluster epithelium. Finally, mitotic cell rounding within the pressurized regions cause a patterned series of folding events that demarcate villi. This process allows efficient and faithful transmission of a mesenchymal pattern into the epithelium.

Furthermore, our exploration of the villus phenotype in the Ezrin null mouse model has revealed several novel aspects of that phenotype that have also impacted our understanding of normal villus morphogenesis. We established for the first time that the Ezrin null epithelium is stratified, while the wild type epithelium remains a single layer throughout villus morphogenesis. We identified altered spindle angle as a mechanism that can both generate this stratification and set the stage for the formation of ectopic lumens seen in that model. Finally, we present the first functional evidence of junctional stability in the Ezrin null intestine that could allow for formation of fused villi that are seen in the perinatal and adult stages. These studies, combined with recent work on the Crb3 null mouse model (Whiteman et al., 2014), reveal that apical surface proteins are critically important in the proper shaping of intestinal villi.

The computational model of the developing epithelium that we have developed will be a valuable tool for further exploration of how forces and signaling intersect in tissue morphogenesis. Expanding this model to view the system as a 2D plane will improve our understanding of how multiple clusters interact with one another. Further evolution of the model
to encompass the 3D shape of the intestine may allow us to incorporate active tissue forces, such as peristalsis, thereby yielding further insights as to how the surrounding cell layers affect fold formation in the developing intestine.
Figure IV-1. TGFβ signaling affects villus morphogenesis downstream of the ELF3 transcription factor. (A) TGFBRII is expressed in both the epithelium and mesenchyme. (B) Epithelial expression is specifically lost in Elf3−/− mice. (C-E) Perturbed villus morphology at E18.5 in (D) Elf3−/− compared to (C) wild type mice. (E) Architecture is restored after expression of the TGFBRII transgene in the intestinal epithelium. (F-H) In situ hybridization for genes encoding TGFβ ligands, Tgfb1, Tgfb2, and Tgfb3, in the small intestine at E14.5. All have mesenchymal expression, although Tgfb3 (H) may be specifically localized to mesenchymal clusters (arrowheads). Scale bar = 100 µm. (Adapted with permission from Ng et al., 2002; Flentjar et al., 2007; genepaint.org)
**Figure IV-2.** *Ex vivo* Dorsomorphin treatment affects formation of sharp apical folds. Intestines were harvested at E14.5 and cultured for two days in the presence of DMSO (A, B) or 20 µm Dorsomorphin (C, D). For comparison, an uncultured intestine at E15.5 (E, F) is pictured. Note that initial indentations (arrowheads) formed in the distal regions in the presence of Dorsomorphin, although the demarcations between villi are much less sharp (compare C to A and F, arrows), suggesting that apical folds did not form with this treatment. The very sharp demarcations in the uncultured intestine (compare A to F, arrows) suggest that culturing *ex vivo* has an effect on formation of sharp folds, even in the absence of small molecule treatment. Scale bar = 50 µm.
Figure IV-3. Wnt signaling is active in inter-cluster and intervillus regions early in villus morphogenesis. (A-C) In situ hybridization for Axin2 at (A) E14.5 (B, C) and E16.5. S = stomach, D = duodenum, IV = intervillus region. Note the absence of Axin2 signal at E14.5 but robust signal at E16.5 restricted to the intervillus regions (arrowhead). (D, E) Serial sections of the intestine at E14.5, with initial clusters forming (C = cluster) and deforming the overlying epithelium (E-cadherin, red). CD44v6 (green) is localized to the inter-cluster epithelium and along the edge of the section, where there is a sharp turn in the epithelium (arrowhead). (Adapted with permission from Li et al., 2009)
Figure IV-4. Preliminary evidence that daughter cells of some divisions at folds segregate onto adjacent villi. (A) Pair of recently divided cells (pHH3, purple) adjacent to a short apical fold (phalloidin, red). Scale bar = 5 μm. (B) In some cases, the cytokinetic plane between two divided cells is apically polarized (EZRIN, green). Scale bar = 2 μm. (C) Schematic for lineage tracing studies of Shh^{CreER^{+}+/ROSA}^{Confetti/+/} mice. (D) Clone size increased with increasing induction time from 24 to 48 hours. (E) Distribution of multi-cell clones between one villus and two villi with 24 and 48 hour induction. Note the relative consistency between these values, in spite of the increased clone size. (F, G) 3D reconstructions of cell clones restricted to one (F) or two (G) villus domains. Scale bar = 20 μm. (H, I) Midbody (α-tubulin, green) orientation in cell dividing adjacent to a flat apical surface (aPKC, red) (H) compared with a cell dividing at a small fold (I). Note the bent versus straight orientation of this structure, suggesting cytokinesis occurs differently in these divisions. Scale bar = 5 μm.
Figure IV-5. Treatment with aPKC-PS causes an ectopic fold phenotype. Treatment of (A, B) wild type or (C, D) $Ez^{ShhKO}$ intestines with 80 µM aPKC-PS for 48 hours results in many ectopic folds, but only in the wild type intestine (B). The $Ez^{ShhKO}$ intestine does not exhibit ectopic fold formation (D). Scale bar = 50 µm.
Figure IV-6. Potential mechanisms for ectopic lumen formation in the Ezrin null intestinal epithelium. Ectopic lumens, disconnected from the apical surface, may form by cell division-independent or cell division-associated mechanisms. In both cases, the origin of the defect lies in the perturbed spindle angle of mitosis. (A) In a cell division-independent mechanism, an initial oblique division can cause a stratified epithelium to result. Within one of these cells disconnected from the apical surface, apical components (red dots) could form an initial rosette along the boundary between two cells. (B) In a cell division-associated mechanism, the initial oblique division may concurrently form a new lumen (red). (C) Alternatively, within the partially stratified epithelium, one of the cells that lacks a contact with the apical surface may undergo mitosis at a location distinct form the apical surface, which may form a lumen concurrently with cell division.
Literature Cited


