Single continuous lumen formation in the zebrafish gut is mediated by smoothened-dependent tissue remodeling

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ABSTRACT
The formation of a single lumen during tubulogenesis is crucial for the development and function of many organs. Although 3D cell culture models have identified molecular mechanisms controlling lumen formation in vitro, their function during vertebrate organogenesis is poorly understood. Using light sheet microscopy and genetic approaches we have investigated single lumen formation in the zebrafish gut. Here we show that during gut development multiple lumens open and enlarge to generate a distinct intermediate, which consists of two adjacent unfused lumens separated by basolateral contacts. We observed that these lumens arise independently from each other along the length of the gut and do not share a continuous apical surface. Resolution of this intermediate into a single, continuous lumen requires the remodeling of contacts between adjacent lumens and subsequent lumen fusion. We show that lumen resolution, but not lumen opening, is impaired in smoothened (smo) mutants, indicating that fluid-driven lumen enlargement and resolution are two distinct processes. Furthermore, we show that smo mutants exhibit perturbations in the Rab11 trafficking pathway and demonstrate that Rab11-mediated trafficking is necessary for single lumen formation. Thus, lumen resolution is a distinct genetically controlled process crucial for single, continuous lumen formation in the zebrafish gut.

KEY WORDS: Lumen, Remodeling, Tubulogenesis

INTRODUCTION
Tubulogenesis is a crucial process during the formation of many organs, including the pancreas, lungs, vasculature, mammary gland and gut. Tube formation mechanisms are diverse across organ systems, but they all result in a structure with a single lumen. Tubes arising from a polarized epithelium typically undergo a process of epithelial wrapping or budding that is driven primarily by changes in cell shape. By contrast, tubes originating from unpolarized cells form through a process of cord hollowing or cavitation that requires the establishment of cell polarity and de novo lumen formation (Lubarsky and Krasnow, 2003; Martin-Belmonte and Mostov, 2008). Lumen formation occurs through the coordinated effort of several distinct cellular processes, including intracellular trafficking to a prospective apical domain, de novo apical membrane biogenesis, lumen enlargement and, in many cases, epithelial remodeling.

De novo lumen formation is integral to the development of tubules that form from an unpolarized epithelium and has been extensively studied in vitro in 3D cysts. To initiate lumen formation, apical membrane proteins such as Podoclyxin accumulate in Rab11 and Rab8a-positive vesicles. These vesicles are then delivered to the plasma membrane where, together with the exocyst and the Par3 complex, they fuse to generate an apical surface (Bryant et al., 2010). Although these studies highlight the importance of apical membrane trafficking in lumen formation, such in vitro systems cannot fully recapitulate the complexity of a three-dimensional organ. For example, in most 3D cyst models the lumen typically forms between two differentiated epithelial cells by recycling of apical membrane components from the surface to the new lumen (Bryant et al., 2010). Thus, lumen formation in vitro does not require processes of epithelial transformation and remodeling that are necessary for tube formation in vivo in many organs. Because of these differences, the cellular mechanisms controlling lumen formation in large unbranched tubes, particularly in vertebrates, remain poorly understood.

The zebrafish intestine begins as a solid rod of endodermal cells that differentiate into epithelial cells and undergo a cord hollowing process to form a tube. Lumen formation initiates with the development of multiple actin-rich foci between cells and is followed by the localization of junctional proteins at multiple points within the intestine (Horne-Badovinac et al., 2001). Small lumens form at these points and expand, coalesce and eventually form a single continuous lumen (Bagnat et al., 2007). Interestingly, intestinal villus formation in the rat epithelium may also form through fusion of small secondary lumens (Madara et al., 1981). Previous work in zebrafish showed that paracellular ion transport regulated by Claudin15 and the Na⁺/K⁺-ATPase drives fluid accumulation, promoting lumen expansion and coalescence in to a single lumen (Bagnat et al., 2007). However, as the gut lumen forms without apoptosis (Ng et al., 2005), other cellular processes such as epithelial remodeling must occur to facilitate lumen coalescence.

Here we provide a high-resolution in vivo characterization of lumen formation in the zebrafish gut. Using this approach we identified a crucial stage in lumen coalescence that is regulated by smoothened (smo) signaling. We show that single, continuous lumen formation starts with rapid lumen expansion in an anterior to posterior (AP) manner. Expansion is followed by an intermediate structure characterized by adjacent unfused lumens. These lumens fuse through both luminal membrane expansion and the loss of adhesion at the fusion site. We also found that smo mutants are unable to undergo the crucial process of lumen fusion and thus fail to form a single continuous lumen in the gut. Furthermore, we show smo mutants exhibit altered localization of Rab11a and demonstrate that proper Rab11-mediated trafficking is important to the formation of a single continuous lumen in the zebrafish intestine.
RESULTS

Single lumen formation involves a distinct double lumen intermediate

Lumen formation in the zebrafish gut begins with the appearance of multiple small lumens that enlarge through fluid accumulation and coalesce to form a single lumen (Bagnat et al., 2007). However, fluid accumulation alone cannot drive the cellular rearrangements necessary for lumen coalescence. The complexity of these processes suggests that other processes are required. To elucidate the process of lumen formation in the zebrafish intestine we performed a timecourse analysis from 48 hours post fertilization (hpf) to 72 hpf and characterized the appearance of the lumen at 4-hour intervals. Analysis of fixed, thick transverse sections by confocal microscopy revealed a range of lumen morphologies. We classified the intestinal tubes into three categories: class I, containing multiple small lumens, in which two to four actin foci or small lumens span the intestine (Fig. 1A); class II, represented by enlarged, unfused lumens, in which a bridge of cells separate open lumens (Fig. 1B); class III, single lumens, characterized by one enlarged continuous lumen (Fig. 1C). In 48 hpf embryos, all three lumen types are apparent with relatively similar frequency. Class I and class III lumens were found in 38% of embryos, whereas class II lumens were found in 24% of gut sections. Over the next 12 hours the appearance of class I lumens decreased, whereas the frequency of class II lumens increased to 30% and class III lumens increased to 70% at 60 hpf. During the subsequent 12 hours, the number of embryos with class II lumens decreased and by 68 hpf only single lumen guts were observed (Fig. 1D). Thus, single lumen formation is preceded by two stereotypic luminal arrangements that include both multiple small lumens and enlarged, unfused lumens.

The most frequently observed luminal arrangement before single lumen formation is two enlarged lumens. However, this arrangement is not simply the result of two parallel lumens spanning the intestine. Using Imaris imaging software, we generated a 3D rendering of lumen size and shape from a 200-µm transverse confocal stack.

Even within this small region, lumens are discontinuous and highly dynamic in shape and size (Fig. 1E). To gain a better understanding of how these discontinuous lumens are arranged along the AP axis we performed whole-mount confocal imaging. Analysis of the anterior intestinal bulb at 58 hpf revealed two enlarged lumens side by side (Fig. 1F), which is representative of the unfused lumens (class II) we observed in transverse cross section. These adjacent, arranged lumens are most frequently observed in the anterior gut, probably owing to the larger diameter and number of cells in this region. Enlarged discontinuous lumens were found along the AP length of the intestine. Toward the posterior end of the intestine, discontinuous lumens were more abundant and of smaller size (Fig. 1G). The unfused lumen phenotype (class II) represents a previously uncharacterized stage in normal lumen formation, and we have termed this phase the ‘lumen resolution stage’. Together, these data suggest that lumen formation occurs through stages of multiple small, and expanded unfused lumens before resolving into a single continuous lumen.

Rapid lumen expansion and fusion during tubulogenesis

Analysis of fixed tissue sections suggested that initial lumen expansion and lumen resolution are distinct phases of intestinal lumen formation. As sectional analysis only offers a static snapshot of lumen formation, we wanted to monitor lumen formation in the intestine using live imaging. To image lumen coalescence in vivo, we required a new transgenic line that is intestine-specific and expresses before single lumen formation. To identify intestine-specific genes we isolated intestinal epithelial cells using a Tg(−1.0ifabp:GFP-CaaX) line which expresses membrane green fluorescent protein (GFP) in the intestinal cells starting around 120 hpf. Using RNA isolated from these cells we performed a microarray analysis and found that one of the most highly intestine-enriched genes was claudin 15-like 1 (cldn15la) (data not shown), a member of the Claudin family of tetraspanning membrane proteins (Furuse et al., 1998). By in situ hybridization, cldn15la was highly expressed before single lumen formation. To examine cldn15la expression during intestinal morphogenesis, we performed whole-mount RNA in situ hybridization at 48, 52, 56, 60, 64, and 68 hpf. Using a probe specific for cldn15la, we performed fluorescent in situ hybridization experiments on embryos at these time points. Our results confirmed that cldn15la expression is restricted to the intestinal epithelium during intestinal development and suggests a role for this gene in intestinal morphogenesis. Even within this small region, lumens are discontinuous and highly dynamic in shape and size (Fig. 1E). To gain a better understanding of how these discontinuous lumens are arranged along the AP axis we performed whole-mount confocal imaging. Analysis of the anterior intestinal bulb at 58 hpf revealed two enlarged lumens side by side (Fig. 1F), which is representative of the unfused lumens (class II) we observed in transverse cross section. These adjacent, arranged lumens are most frequently observed in the anterior gut, probably owing to the larger diameter and number of cells in this region. Enlarged discontinuous lumens were found along the AP length of the intestine. Toward the posterior end of the intestine, discontinuous lumens were more abundant and of smaller size (Fig. 1G). The unfused lumen phenotype (class II) represents a previously uncharacterized stage in normal lumen formation, and we have termed this phase the ‘lumen resolution stage’. Together, these data suggest that lumen formation occurs through stages of multiple small, and expanded unfused lumens before resolving into a single continuous lumen.

Fig. 1. Lumens enlarge and fuse during single lumen formation in the zebrafish gut. (A-C) Confocal images of cross sections of wild-type embryos exhibiting class I (A), class II (B) and class III (C) lumens, stained with phalloidin. Arrowheads indicate the lumens. (D) Quantification of lumen phenotypes between 48 and 72 hpf: 48 hpf n=21, 52 hpf n=29, 56 hpf n=27, 60 hpf n=27, 64 hpf n=30, 68 hpf n=21, 72 hpf n=26. (E) Space-fill projection from a 200 µm confocal stack of an intestine section at the resolution stage. Yellow, lumen; green, GFP-CaaX; blue, DAPI. (F) Confocal whole-mount image of the anterior gut at 58 hpf stained with phalloidin (red). Arrowheads point to adjacent unfused lumens. (G) Confocal whole-mount image of the posterior gut at 58 hpf stained with phalloidin (red). Arrowheads point to lumens. Scale bars: 20 µm in A-C; 10 µm in E; 20 µm in F,G.
expressed and restricted to the intestine by 50 hpf (Fig. 2A,B). To generate a transgenic line expressing Cldn15la-GFP we used bacterial artificial chromosome (BAC) recombineering to create a C-terminal fusion protein (Fig. 2C).

Cldn15la-GFP expression was first observed at 48 hpf in the intestinal epithelium and remained expressed throughout the course of lumen formation (Fig. 2G,H). An analysis of transverse sections revealed that Cldn15la-GFP is restricted to the intestine and is not expressed in other endoderm-derived organs (Fig. 2D). Cldn15la-GFP was found localized to the lateral surface of the intestinal epithelium (Fig. 2E). Although Claudin proteins are components of tight junctions and typically localize to the subapical region (Furuse et al., 1998), studies have shown that several members of this protein family also localize to lateral membranes during morphogenesis (Gregory et al., 2001; Inai et al., 2007; Westmoreland et al., 2012), including the closely related zebrafish claudin Cldn15lb (Cheung et al., 2012). To determine whether Cldn15la-GFP lateral membrane localization represented the endogenous protein localization we generated an antibody against the C-terminus of Cldn15la. Similar to the BAC transgenic construct, Cldn15la localized to the lateral membrane in intestinal epithelial cells, indicating that the Cldn15la-GFP fusion recapitulates endogenous expression and localization (Fig. 2F). Furthermore, we generated additional transgenic lines with a different linker sequence between GFP and Cldn15la and observed a similar localization pattern (data not shown). The Cldn15la-GFP transgene allowed for improved examination of the cellular and luminal arrangements within the intestine. Whole-mount imaging of the entire intestine revealed that lumen fusion begins in the anterior region and proceeds in an anterior to posterior direction (Fig. 2M). In addition, we observed that unfused lumens were frequently separated by single cell-cell contacts (Fig. 2N).

To visualize the process of lumen formation live, we used selective plane illumination microscopy (SPIM) (Huisken and Stainier, 2009) to image TgBAC(cldn15la-GFP) embryos (supplementary material Movies 1, 2). Initially, several small lumens were seen opening along the AP length of the intestine (Fig. 2J). These lumens were often separated by a few cells, which is similar to those observed in fixed whole-mount embryos at the resolution stage. Initially, the expansion of these lumens was rapid and followed by local fusion events that resulted in two to three large luminal compartments. The larger lumens remained separated by a one- or two-cell-thick cellular bridge for an extended period of time, yielding a distinct intermediate (Fig. 2K). Ultimately, these large lumens resolve into one (Fig. 2L). Taken together, our morphological and live imaging studies reveal that single lumen formation in the zebrafish intestine involves two distinct morphological and kinetic phases and identify a previously unknown stage characterized by the presence of large, unfused lumens.

**Basolateral adhesions localize to cell-cell contacts between lumens**

As lumen coalescence occurs in the absence of apoptosis (Ng et al., 2005), processes must be involved to facilitate tissue remodeling during lumen resolution. To address the process of lumen fusion, we further characterized the resolution stage. Analysis of the cellular architecture of transverse intestinal sections at the resolution stage using a membrane GFP marker, Tg(hsp70l:GFP-CaaX)pd1008, revealed that lateral lumens were often separated by a bridge of cells the contacts of which form a Y- or T-shaped arrangement between two adjacent lumens (Fig. 3A,A′). To determine the identity of the bridge contacts, we examined the localization of specific apical and basolateral proteins. Using a Tg(hsp70l:GFP-podxl)pd1080 line, we...
found that the apical membrane protein Podocalyxin localized to the apical surface surrounding the lumens and was absent from the connecting bridge (Fig. 3B). Similarly, the tight junction protein ZO-1 (Tjp1a – ZFIN) was restricted to the subluminal area and was not found at the membrane between lumens (Fig. 3C). By contrast, the adhesion proteins cadherin and β-catenin were localized to all basolateral membranes and were also located on the bridge membrane separating the two lumens (Fig. 3D,E). These data reveal that during the resolution stage, cells surrounding the lumens are polarized and adjacently arranged lumens within an intestinal cross section are separated by basolateral contacts that exclude apical proteins.

We next examined the process of lumen resolution along the length of the intestine. The generation of a single continuous lumen is a more complex process involving the coordination of several lumens along the gut. There are two possible scenarios in which a single continuous lumen can resolve from multiple discontinuous lumens (Fig. 3F). One possibility is that apical membrane can be deposited at bridge contacts between lumens, forming a continuous path to connect the enlarging lumens. Alternatively, each lumen may be an autonomous unit separated by basolateral contacts, similar to adjacently arranged lumens. In this case, single lumen formation would require the disengagement of the cell-cell contacts between adjacent lumens. To determine which scenario most accurately represents the process of lumen resolution along the intestine we performed whole-mount analysis of Tg(hsp70l:GFP-podxl)pd1080 embryos stained for cadherin. Consistent with the transverse section data, lumens along the AP axis were frequently separated by Y- and T-shaped cadherin-positive contacts and GFP-Podxl was restricted to the membrane surrounding the lumens (Fig. 3G-G′). Thus, the organization of adjacent lumens seen in transverse sections is analogous to the organization of adjacent lumens along the AP axis. Furthermore, we found no evidence of apical membrane deposition between two lumens before lumen fusion.

Lumen resolution may occur via the expansion and direct fusion of luminal membranes, or through the reduction and breaking of contacts between the lumens or both. Before lumen fusion adjacent lumens expand and the connecting bridge appears to shrink. We observed that in regions where the basolateral bridge contact was particularly narrow, GFP-podxl-positive membranes protruded from the adjacent luminal surfaces toward a central area with diffuse cadherin signal, probably originating from the internalization of the contact (Fig. 4A-A′; supplementary material Movie 3). We termed this type of resolution event ‘luminal fusion’. Further analysis of cadherin-stained and TgBAC(GFP-cldn15la) embryos revealed that in some instances during the fusion process, cadherin and GFP-Clnd15la can still be found at the fusion site. Although the basolateral proteins are not completely removed, cell-cell adhesion is lost. This localization at the cell surface probably originates from the separated bridge contact, suggesting that the adhesions between the cells had snapped before their complete internalization (Fig. 4C-F). Resolution through luminal fusion seems to be the predominant mode (65%), whereas snapping accounted for 35% of the events (n=20). These data indicate that lumen resolution involves remodeling of bridge contacts through both apical membrane expansion and the reduction of the adhesion contact.

**smoothened mutants exhibit impaired lumen fusion**  
We next sought to identify a genetic model to investigate the resolution stage of lumen formation in the intestine. The hedgehog (Hh) pathway is known to be involved in gastrointestinal tract morphogenesis in mammals and cloaca formation in zebrafish.
Therefore, we examined lumen formation in embryos mutant for *smoothened* (*smo*), the hedgehog co-receptor. We performed transverse sectional analyses of homozygous *smo*{superscript 294} (Aanstad et al., 2009) mutant embryos at 72 hpf, a time point when a single continuous lumen is well established in wild-type embryos. The *smo*{superscript 294} allele contains a mutation in a conserved cystine residue in the extracellular domain of the protein and is essential for full activation of the Hh pathway (Aanstad et al., 2009). At 72 hpf, ~43% of *smo*{superscript 294} mutant embryos (n=21 mutants) exhibit impaired intestinal lumen fusion (Fig. 5A,E). To confirm that the smoothened mutation is responsible for the lumen formation defect, we examined a null allele of *smoothened*, *smohi1640* (Chen et al., 2001) and found the same phenotype in a similar proportion of embryos (44%, n=27) (data not shown). The *smo*{superscript 294} phenotype was similar to the class II wild-type intermediate, which indicates a failure at the resolution stage. To determine if unfused lumens resolve at a later time in development, we also examined embryos beyond 72 hpf. The unfused lumen phenotype was observed at 85 hpf, 96 hpf and 110 hpf (Fig. 5B-F). At 96 hpf 27% of *smo*{superscript 294} embryos (n=26 mutants) continued to exhibit unfused lumens, indicating that impaired lumen fusion in mutants is not due to a developmental delay. In addition to transverse sectional analysis, we also examined *smo*{superscript 294} in whole mount to determine if impaired fusion was displayed along the entire intestine or was restricted to the anterior intestinal bulb. At 72 hpf, *smo*{superscript 294} mutants exhibited several unfused lumens along the intestine (Fig. 5I-J), which is consistent with the results observed in wild-type embryos at the resolution stage. It is important to note that in *smo*{superscript 294} mutants, unfused lumens are fully open and continue to expand as cells divide (Fig. 5E-H). These lumens can be distinguished from the surrounding wild-type lumen by their size and shape. The unfused lumens are larger and more irregular in shape compared to the wild-type lumen. This difference in morphology suggests that the unfused lumens are not simply a result of incomplete fusion, but rather represent a distinct developmental stage. In summary, these results provide strong evidence that the *smo*{superscript 294} mutation disrupts lumen formation by impairing the resolution stage of intestinal development. Further studies will be necessary to determine the specific molecular mechanisms underlying this defect.
results indicate that the smo<sup>294</sup> phenotype results from a failure in lumen resolution and not from impaired fluid accumulation. Together, these data support the idea that fluid alone cannot drive single lumen formation and reveal that lumen opening and lumen fusion are two distinct events required for single lumen formation that can be genetically uncoupled.

To examine the spatiotemporal expression of smo we used a transgenic reporter line for the Hh pathway target gene patched (Choi et al., 2013). At 48 and 72 hpf signaling was observed in the mesenchyme surrounding the gut (supplementary material Fig. S1A,B). Hh signaling is known to play an important role in the differentiation of mesodermal precursors into smooth muscle. In smo<sup>294</sup> the mesenchymal layer contains fewer, more elongated cells compared with wild type (Fig. 5E-H). Therefore, we examined the differentiation of the mesenchymal layer in smo mutants. In situ hybridization revealed that expression of the smooth muscle marker αSMA is lacking in mutant embryos at 72 hpf, indicating an absence of differentiated smooth muscle surrounding the gut (supplementary material Fig. S1D,E).

To determine if impaired lumen fusion in smo mutants is due to an early endoderm migration defect, we examined expression of the endoderm marker, foxa3. At 30 hpf smo mutants show a single, midline localized endodermal rod that is overall similar in shape to that of wild-type embryos (supplementary material Fig. S2A,B). Examination of TgBAC(cldn15la-GFP) embryos at 48 hpf revealed that the intestinal epithelium is also similar in size and shape in wild type and smo mutants (supplementary material Fig. S2C,D,G). Furthermore, we determined that there is no significant difference in cell number, or cell proliferation between wild type and smo mutants (supplementary material Fig. S2E-F,H,I). There was also no observable apoptosis in the gut of wild-type or smo embryos (data not shown). Therefore, the lumen fusion phenotype observed in smo mutants does not result from defects in early endoderm migration, or impaired regulation of epithelial cell numbers.

**Rab11-mediated recycling is misregulated in smo<sup>294</sup> mutants**

Because lumen formation in the zebrafish gut occurs without apoptosis (Ng et al., 2005), lumen resolution must involve epithelial remodeling and the rearrangement of cellular contacts between lumens. This remodeling can be achieved by changing the identity of bridge contacts, from basolateral to apical, or by breaking adhesions. To undergo remodeling, cellular contacts and adhesions can be internalized and trafficked to lysosomes for degradation or they can be recycled back to the cell surface (Le et al., 1999; Palacios et al., 2005). To determine if lysosomal degradation is important for lumen fusion, we inhibited the degradation pathway by expressing a dominant-negative form of Rab7, a small GTPase that regulates late endosomal trafficking (Bucci et al., 2000). We also inhibited lysosomal acidification using bafilomycin, and examined embryos mutant for members of the vacuolar H<sup>-</sup>-ATPase complex (Nuckels et al., 2009). However, no lumen fusion defects were observed (supplementary material Fig. S3A-F).

Several studies have found that endocytic recycling and trafficking are important for epithelial remodeling during morphogenesis. The Rab11 family of small GTPases as well as the Rab11 effector proteins Rab11-FIP and MyoVb are well-known regulators of the recycling pathway (Hales et al., 2001; Lapierre et al., 2001; Ulrich et al., 1996) and play a key role in apical trafficking and basolateral recycling during epithelial morphogenesis (Kerman et al., 2008; Satoh et al., 2005; Shaye et al., 2008). To determine if recycling is involved in single lumen formation, we utilized a dominant-negative construct to disrupt endogenous Rab11a function. We crossed Tg(UAS:mcerry-rab11a-S25N)mw35 (Rab11aDN) (Clark et al., 2011) to a Tg(hsp70:gal4) line to temporally control expression of Rab11aDN. Mosaic expression of Rab11aDN before the resolution stage of lumen formation resulted in an unfused lumen phenotype, similar to that of smo<sup>294</sup> mutants and class II wild-type embryos, in 45% of embryos (n=20) at 72 hpf (Fig. 6A). These embryos contained the same number of epithelial cells in the gut as wild-type embryos, indicating that failed lumen resolution is not due to differences in cell numbers (supplementary material Fig. S5A). Upon expression of Rab11aDN, cadherin accumulated intracellularly, indicating that it is recycled in a Rab11a-dependent manner (supplementary material Fig. S4A-E). In addition, the apical protein 4e8 was also found to colocalize to Rab11aDN compartments (supplementary material Fig. S4F-J). We also tested the function of Rab11b, which is highly similar to Rab11a, yet resides in distinct apical vesicles in epithelial cells and colocalizes with different cargo proteins (Lai et al., 1994; Lapierre et al., 2003). Unlike DN-Rab11a, expression of DN-Rab11b did not cause a lumen formation phenotype (Fig. 6B). Thus, Rab11a-mediated recycling of basolateral and apical membrane proteins is necessary for lumen fusion during single lumen formation.

To determine if Rab11aDN embryos exhibit a mesenchymal differentiation defect similar to that of smo mutants, we examined the expression of αSMA. Expression of αSMA in Rab11aDN embryos revealed proper differentiation of the mesenchymal layer (supplementary material Fig S5B,C). Furthermore, staining for smooth muscle myosin, Myh11, showed that mesenchymal cells expressing Rab11aDN differentiated as well as the controls (supplementary material Fig. S5D,E), suggesting that Rab11aDN expression does not affect the differentiation of the gut mesenchymal layer and that the lumen resolution phenotype was not due to mesenchymal defects.

The similar lumen phenotype shared by smo<sup>294</sup> mutants and Rab11aDN-expressing embryos next led us to investigate whether defects in the recycling pathway contribute to the smo<sup>294</sup> phenotype. To this end we generated a GFP-Rab11a transgenic line, Tg(hsp70:GFP-RAB11a)pdI031, to mark recycling endosomes. In wild-type embryos, GFP-Rab11a was localized to small subapical compartments surrounding the lumen (Fig. 6C). By contrast, smo<sup>294</sup> mutants exhibited abnormally enlarged GFP-Rab11a compartments that were dispersed from the apical surface (Fig. 6D). These enlarged Rab11a compartments in smo mutants contained the apical protein 4e8, indicating a defect in trafficking of apical membrane proteins (Fig. 6E,F). Colocalization with cadherin was not apparent (Fig. 6G,H); however, owing to the transient nature of internalized cadherin, cadherin colocalization with recycling endosomes is often limited (Deslozeaux et al., 2008). This probably accounts for the minimal amount of colocalization with Rab11a compartments we observe. These data, together with the Rab11DN data, suggest that Rab11 trafficking of both apical and basolateral proteins is important in lumen fusion.

Previous studies in the Drosophila trachea have shown a similar accumulation of Rab11 in enlarged compartments upon overexpression of the Rab11 effector protein Rip11, an ortholog of Rab11Fip1a (Shaye et al., 2008). Rab11Fip1a and MyoVb interact with Rab11 family members and regulate plasma membrane recycling (Hales et al., 2001; Lapierre et al., 2001). To investigate Rab11 effectors in smo<sup>294</sup> mutants we examined the expression levels of Rab11a, Rab11b, Rab11Fip1a and MyoVb in wild-type and smo<sup>294</sup> embryos. We used fluorescence-activated cell sorting (FACS) to isolate intestinal cells from mutant TgBAC(cldn15la-
GFP
d1034; smos294 embryos and wild-type clutchmates, isolated RNA and performed quantitative polymerase chain reaction (qPCR) to evaluate differential gene expression. In smos294 cells, expression of rab11fip1a was increased threefold. However, the expression of rab11a, rab11b and myo5b was not significantly different from that in wild-type cells (Fig. 6K). To assess how an increase in Rab11fip1a levels may contribute to the smo gut phenotype, we overexpressed Rab11fipa1 in Tg(hsp70l:GFP-RAB11a) embryos through RNA injection. Upon mild overexpression, GFP-Rab11a compartments became enlarged and disorganized compared with non-injected embryos, similar to that observed in smo mutants (Fig. 6L,J). The data suggest that increased levels of Rab11fip1a are likely to be responsible for the abnormally enlarged GFP-Rab11a compartments observed in smo294 mutants.

Altogether, these studies identify an intermediate stage in the process of single lumen formation and reveal that lumen resolution is a genetically regulated process crucial for continuous lumen formation in the zebrafish gut. Our data also highlight the role of smothened signaling from the mesenchyme in the regulation of lumen morphogenesis in the gut epithelium.

**DISCUSSION**

In this study we identify lumen resolution as a crucial process during single lumen formation. Single lumen formation begins with multiple small lumens that enlarge through fluid accumulation driven by ClC15 and Na+/K+ ATPase (Fig. 7A). Before lumen coalescence, enlarged lumens are found along the length of the gut, separated by basolateral bridge contacts. Our studies reveal that cell-cell bridge contacts lack apical and tight junction markers between lumens, indicating that these bridge contacts do not change identity before lumen fusion. Instead, we observed that lumen fusion occurs through both apical membrane expansion and the shrinking and breaking of basolateral bridge contacts. The most common bridge cell arrangement involves cells that have one apical surface; however, occasionally bridge cells exhibit two apical surfaces. Bipolar cells have also been observed during tubulogenesis in the Ciona notochord (Denker and Jiang, 2012). Although the mechanism by which a cell acquires two apical membranes is unknown, it is possible that the cells between lumens are unable to receive proper polarizing cues from the basement membrane.

Based on our studies, we propose that Smo signaling facilitates the remodeling and weakening of bridge contacts and the enlargement of apical membrane via Rab11a-mediated trafficking and recycling to generate a single continuous lumen (Fig. 7A). In this model basolateral recycling relocalizes the adhesion from the bridge to lateral surfaces, thus shrinking and weakening the contacts between lumens. In addition, apical membrane is delivered to the luminal surface to facilitate membrane expansion. As adhesions shrink, the bridge
contacts eventually break and lumens fuse (Fig. 7B). This may also be facilitated by the insertion of anti-adhesive apical proteins on the edge of the bridge contact, as shown in blood vessels (Strilić et al., 2010). Interestingly, zebrafish mutant for aPKcλ, which regulates adherens and tight junctions, also exhibit a single lumen formation defect (Horne-Badovinac et al., 2001), underscoring that proper regulation of adherens is critical in single lumen formation.

Work in 3D cysts has established the importance of functional Rab11 and recycling endosomes in E-cadherin trafficking, cyst morphogenesis and lumen formation (Bryant et al., 2010; Desclozeaux et al., 2008). In 3D cysts, Rab11 is crucial for lumen initiation by mediating the relocation of apical membrane from the outer surface of cells to a central patch where a lumen subsequently forms (Bryant et al., 2010). However, in most in vivo tubular systems, including the zebrafish intestine, mammalian pancreas and mammary gland, lumens initiate at several different sites within a rod of cells and must connect with each other to form a continuous luminal network. Our work shows that Rab11-mediated trafficking is needed to facilitate lumen resolution. Therefore, Rab11 may play a role in two distinct processes of lumen formation: lumen initiation and lumen resolution.

During the resolution stage lumens may merge through direct apical-apical membrane fusion or through fusion at cell junctions. Studies in the zebrafish vasculature and ascidian notochord provide insight into the possible mechanisms involved in membrane and junctional coalescence during lumen fusion. During ascidian notochord tubulogenesis, cellular remodeling involves a reduction of intracellular junctions between neighboring cells and the establishment of a new junction between two previously unconnected cells (Dong et al., 2009). Furthermore, work in the zebrafish dorsal longitudinal anastomotic vessel suggests that two apical membranes can merge directly (Herwig et al., 2011). However, a more detailed study will be needed to determine whether lumens merge through apical fusion in the zebrafish gut.

Examination of recycling pathway members revealed differential expression of the Rab11 effector protein, Rab11fip1a, and an accumulation of enlarged Rab11a compartments in smo294 intestinal epithelium. Overexpression of Rab11fip1a caused a similar accumulation of Rab11a compartments compared with wild-type embryos but did not produce a lumen fusion phenotype. This suggests that additional genes are also involved or that higher levels of Rab11fip1a expression are required to cause a lumen formation defect. In the Drosophila trachea, the overexpression of the Rab11 effector protein, Rip11, causes an accumulation of large Rab11 vesicles, similar to that observed in smo294 mutants, and results in impaired morphogenesis (Shaye et al., 2008). Therefore, our findings in smo294 mutants are consistent with previous studies and highlight the importance of effector protein levels in modulating endocytic recycling.

Although smo294 exhibits aberrant Rab11a localization and increased expression of Rab11 effector proteins, it is unclear how smo signaling regulates these recycling pathway members. We found that smo signaling acts in the surrounding mesenchyme but not in the intestinal epithelium. In the zebrafish esophagus and swimbladder, molecular interactions between epithelial Hh and mesenchymal Fgf10 regulate proliferation and differentiation (Korzh et al., 2011). In addition, Hh signaling from the endoderm is required for posterior gut development in zebrafish embryos (Parkin et al., 2009). Thus, smo probably regulates lumen fusion through epithelial-mesenchymal interactions and/or morphogen signaling such as the Bmp or Fgf pathways. Signaling from the mesenchyme through secreted factors and/or mechanical interactions are undoubtedly important for epithelial organization during tubulogenesis. Future studies should dissect the specific role both types of interactions play in regulating gut morphogenesis.

In the zebrafish intestine, lumens open at multiple sites within the gut tube, rather than at a single initiation point as seen in other models of tube formation such as 3D cysts and the C. elegans excretory cell (Bryant et al., 2010; Khan et al., 2013; Kolotuev et al., 2013). We observed that lumens form along the entire length of the gut tube and are typically separated from each other by one or two cells. This architecture allows lumens to fuse through localized cellular rearrangements, thus facilitating the generation of a continuous lumen within a long tube.

MATERIALS AND METHODS

Fish stocks

Zebrafish were maintained at 28°C and bred as previously described (Westerfield, 2000). Zebrafish lines used in this study include: EK, smo294 (Aanstad et al., 2009), Tg(UAS::Cherry-rab11b-52S5N)mw35 (Clark et al., 2011), Tg(hsp70:Gai4) (Scheer et al., 2001), Tg(hsp70:GFP-podxl1080) (Navis et al., 2013), ap66V11b15H72S5N (Navis et al., 2013), ap66V11b15H72S5N (Navis et al., 2013), GBS::pche2::EGFP::umz23 (Clark et al., 2013), TgBAC::cdln15a::GFP::umz23 (Clark et al., 2013), Tg(hsp70:GFP::umz23) (Choi et al., 2013), Tg(hsp70:GFP::umz23) (Choi et al., 2013), Tg(hsp70:GFP::umz23) (Choi et al., 2013), and Tg(hsp70:GFP::umz23) (Choi et al., 2013). In addition, Hh signaling from the endoderm is required for posterior gut development in zebrafish embryos (Parkin et al., 2009). Thus, smo probably regulates lumen fusion through epithelial-mesenchymal interactions and/or morphogen signaling such as the Bmp or Fgf pathways. Signaling from the mesenchyme through secreted factors and/or mechanical interactions are undoubtedly important for epithelial organization during tubulogenesis. Future studies should dissect the specific role both types of interactions play in regulating gut morphogenesis.

In the zebrafish intestine, lumens open at multiple sites within the gut tube, rather than at a single initiation point as seen in other models of tube formation such as 3D cysts and the C. elegans excretory cell (Bryant et al., 2010; Khan et al., 2013; Kolotuev et al., 2013). We observed that lumens form along the entire length of the gut tube and are typically separated from each other by one or two cells. This architecture allows lumens to fuse through localized cellular rearrangements, thus facilitating the generation of a continuous lumen within a long tube.

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Transgenic lines were generated using the Tol2kit (Kwan et al., 2007). Plasmids used include pSE-MCS, pSE-hsp70L, pME-MCS, pME-EGFP-CaaX, p3E-polyA, pDestTol2pA2 and pDestTol2pCG2. pME-RAB11a was generated from Addgene plasmid 12674; GFP-RAB11aWT. pME-GFP-Rab7 and pME-GFP-Rab7DN were generated from Addgene plasmids 12605 and 12660, respectively. Rab11b was amplified from cdNA using primers: Rab11b_Bambi_F, GGATCCATGGGAGCCGTGAGC; Rab11b_NotI_R, GCGGCCGCTACAGTTCTCAGACG and the Rab11bS25N mutation was created using the QuikChange kit (Agilent Technologies).

**BAC recombineering**

A BAC containing cldn15la was modified as previously described (Navis et al., 2013). For the C-terminal GFP fusion, a 20-aa spacer (DLPAEQQKAAEEEDLP) was used. Recombination was performed with homology primers: cldn15a-spGFP_hom_F, CCATCTTATACCA-CAGCTAATGCAAGCAACATCCAAAGGCTACTGCACTCC-CGGCGAAACAGAA, and cldn15a-spGFP_hom_R, TAAACAAACAATCAACGCTACAGTIGTTGTAATATCGGAAATCTGAGTCACGCCCGGGTG. The cldn15a-GFP BAC was linearized using Asell (NEB), injected into one-cell stage embryos to generate TgBAC(cldn15a-GFP)p0d1034.

**RNA injection and BrdU labeling**

pCS2-RFP-Rab11fip1a was linearized using NotI and RNA was transcribed using the mMESSAGE mMACHINE SP6 kit (Ambion). RFP-Rab11fip1a (294 pg) RNA was injected into embryos at the one-cell stage.

To label proliferating cells, 72 hpf embryos were incubated in 16 mM bromodeoxyuridine (BrdU) with 10% dimethyl sulfoxide (DMSO) in egg water for 1 hour at 28°C. Percentage of proliferating cells was calculated by comparing the number of BrdU-positive cells in a gut section versus total cells.

**Histology and immunofluorescence**

Cross sections were cut with a vibratome (VT 1000S; Leica) and stained as described previously (Bagnat et al., 2007). Primary antibodies: pan-cadherin (Sigma; 1:1200), ZO-1 (339100, Invitrogen; 1:500), 4e8 (73643, AbCam; 1:500), β-catenin (SC 7199, Santa Cruz Biotechnology; 1:500), caspase 3 (AB3623, Millipore; 1:500), Myh11 (BT-562, Biomedical Technologies; 1:150) and BrdU (033900, Invitrogen; 1:500). Secondary antibodies (Molecular Probes) were used at 1:300. A custom Cldn15la antibody was generated in rabbit using a peptide derived from the C-terminus of Cldn15la (YQRFSKSKEKGAYYPFC) and used at 1:500. Cldn15la staining was performed as previously described (Dong et al., 2007). Imaging was carried out on an SP5 confocal microscope (Leica, Wetzlar, Germany) with 40×/1.25-0.75 HCX PL APO oil objective, Application Suite (Leica) and Huygens Essential deconvolution software were used for image processing.

**Live imaging**

Zebrafish embryos at 48 hpf were anesthetized and embedded in 1.5% agarose. SPIM was performed using three 10×/0.3 water dipping lenses (Leica), two for illumination and one for detection in an mSPIM configuration (Huisken et al., 2009). The extracellular domain of Smoothened regulates ciliary localization and is required for high-level Hh signaling. Flow Cytometry Shared Resource center (Duke University). Cells were collected in Buffer RLT (Qiagen) and stored at −80°C.

**RNA isolation qPCR**

RNA was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer’s protocol. cdNA was synthesized using First Strand cdDNA Synthesis Kit (Roche). Quantitative PCR was performed using a Bio-Rad CFX96 Real-Time System C1000 Thermocycler and Bio-Rad iQ SYBR Green Supermix. Reactions were performed in duplicate and data from three independent runs were obtained. Primers used include: elfa_F, CTTCCTAGGCTGATCCTGC; elfa_R, CCGTGAATTACCCCTC; myoVb_F, AGGACATGTGGACACCCTTC; myoVb_R, TCCAGCTC-TTGCACCTTTC; rab11fip1a_F, TCAAAACGTTGGGACCATA; rab11fip1a_R, TTTGGGCTTGTGAAGGACAG; rab11a_F, GAAAGAC-CCGTCAAGGCTCG; rab11a_R, ACCTGGATGAGCACACAT; rab11b_F, GAGACAGGAAGCCTACAG; rab11b_R, TGGCCCTT-TAACCCGTACGTA. Expression levels were normalized to elfa for each cdNA set.

**In situ hybridization**

To make an in situ probe, cldn15a was amplified from cdNA using the following primers: cldn probe_F, GGGGCTGGTTGGTTTAGTTT; cldn probe_R, CCGCATCCATGAAAATTGA and ligated into pGEMT-Easy (Promega). The plasmid was linearized and DIG RNA Labeling Kit (Roche) was used to make digoxigenin-labeled RNA. In situ hybridization for cldn15la, foxa3 (Field et al., 2003) and oSMA (Georgijevic et al., 2007) was performed as described previously (Navis et al., 2013) and images were acquired on a Discovery V20 microscope (Zeiss).

**Pharmacological treatment**

Embryos were dechorionated at 48 hpf and placed in a 12-well dish with 1 μM bafilomycin (Sigma) or 1 μM DMSO (Sigma) in egg water. Embryos were incubated at 28°C and fixed at 72 hpf.

**Statistical analysis**

Gut diameter and perimeter was determined using ImageJ software. Number of cells in the gut were obtained by counting nuclei in AP-position-matched sections. Statistical significance for all measurements was determined using Student’s t-test.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

Supplementary material Fig. S1. *smoothened* signaling acts in the surrounding mesenchyme. (A,B) Confocal cross section of the transcriptional reporter *Tg(GBS-ptch2:EGFP)umz23*. Phalloidin (red), DAPI (blue). Scale bar: 20 μm. (C) *Hh* is expressed in the epithelium and binds to *ptch* in the mesenchyme to activate *smo* mediated downstream transcription. Thus, *smo* regulates the epithelium through epithelial-mesenchymal signaling pathways. (D,E) Dorsal view of an *in situ* hybridization showing αSMA expression in WT and *smo* mutant embryos at 72 hpf. Arrows point to smooth muscle. Scale bar: 100 μm.
**Supplementary material Fig. S2.** Gut tube shape and total cell number are similar in wildtype and mutant embryos. (A,B) *In situ* hybridization of WT and smo mutant embryos expressing foxa3 at 30 hpf. Scale bar: 200 µm. (C-D) WT and smo mutant embryo expressing TgBAC(cldn15la-GFP) at 48 hpf. Arrows indicate intestine. (E-F’) Cross section of WT and smo mutant guts at 72 hpf stained for BrdU to label proliferating cells. Scale bars: 20 µm. (G) Quantification of the diameter and perimeter of WT and smo mutant guts from transverse cross sections. WT $n=14$, mutant $n=18$, diameter $P>0.18$, perimeter $P>0.48$. (H) Quantification of total cell number in WT and mutant guts. WT $n=14$, mutant $n=18$, $P>0.30$ (I) Quantification of the percent of BrdU positive cells in WT and smo mutant guts. WT $n=14$, mutant $n=19$, $P>0.32$. 
**Supplementary material Fig. S3. The degradation pathway is not involved in lumen formation.** (A,B) Confocal cross sections from Tg(hsp70l:GFP-Rab7) and Tg(hsp70l:GFP-Rab7DN) embryos. Phalloidin (red). (C,D) Confocal cross sections from DMSO and bafilomycin treated embryos. (E,F) Confocal cross sections from atp6 v1f and atp3 v1e mutant embryos. Phalloidin (red). Scale bars: 20 μm.

**Supplementary material Fig. S4. Apical and basolateral proteins colocalize with Rab11aDN compartments.** (A-E) Confocal cross section of a WT and Rab11aDN embryos at 72 hpf stained for cadherin. Arrowheads point to Rab11DN and cadherin co-localization in internal compartments. (F-J) Confocal cross section of a WT and Rab11aDN embryos at 72 hpf stained for 4e8. Arrowheads point to Rab11DN and 4e8 co-localization in internal compartments. Scale bars: 20 μm.
Supplementary material Fig. S5. Cell number and mesenchymal differentiation is not impaired in Rab11DN embryos. (A) Quantification of total cell number in the gut in WT and Rab11aDN embryos. WT n=13, DN n=11, P>0.32. (B,C) Lateral view of an in situ hybridization showing αSMA expression in WT and Rab11aDN embryos at 72 hpf. Arrow points to smooth muscle. Scale bar: 100 µm. (D-E”) Confocal section of WT and Rab11DN embryo stained for Myh11. Arrowhead points to Myh11 in the mesenchyme. Asterisk indicates non-specific epithelial staining as observed previously (Wallace et al., 2005). Scale bar: 20 µm.

Supplementary material Movie 1. Selective Plane Illumination Microscopy of a TgBAC(cldn15la-GFP) embryo from 48-72 hpf. The images from top to bottom represent different z-planes of a single embryo.
Supplementary material Movie 2. Selective Plane Illumination Microscopy of a TgBAC(cldn15la-GFP) embryo from 56-70 hpf. Small lumens open and fuse along the length of the gut, resulting in two large luminal compartments separated by a cellular bridge. However, this movie ends prior to complete lumen resolution.

Supplementary material Movie 3. Confocal stack of a fixed whole mount Tg(hsp:GFP-podlx) (red) embryo stained with cadherin (green) and DAPI (blue).