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FoxL1^{+} mesenchymal cells are a critical source of Wnt5a for midgut elongation during mouse embryonic intestinal development

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Abstract

Wnt5a is a non-canonical Wnt ligand that is essential for normal embryonic development in mammals. The role of Wnt5a in early intestinal development has been examined in gene ablation models, where *Wnt5a*$^{-/-}$mice exhibit strikingly shortened intestines. However, the exact cellular source of Wnt5a has remained elusive, until a recent study found that FoxL1-expressing mesenchymal cells (FoxL1^{+} cells), which are localized directly beneath the intestinal epithelium, express Wnt5a. To determine whether FoxL1^{+} cells are a required source of Wnt5a during intestinal development, we derived *FoxL1-Cre; Wnt5a*$^{f/f}$ mice, which is the first mouse model to ablate Wnt5a in a cell type-specific manner in the intestine *in vivo*. Our results show that Wnt5a deletion in FoxL1^{+} cells during fetal life causes a shortened gut phenotype in neonatal mice, and that our model is sufficient to increase rate of apoptosis in the elongating epithelium, thus explaining the shortened gut phenotype. However, in contrast to previous studies using Wnt5a null mice, we did not observe dysregulation of epithelial structure or apical-basal protein localization. Altogether, our findings establish a developmental role for FoxL1^{+} mesenchymal cells in controlling non-canonical Wnt signaling during midgut elongation.

Keywords

Intestinal development; Wnt5a; FoxL1; Non-canonical Wnt; Intestinal mesenchyme; Midgut elongation

1. Introduction

The mammalian intestine is essential for nutrient and water absorption, defense against luminal pathogens, and regulation of immune responses. Maintaining structural integrity of the epithelium is required for the intestine to perform its vital functions, and dysregulation

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CRediT authorship contribution statement

KHK conceptualized the study. AK performed all experiments and analyzed the data. AK and KHK wrote the manuscript.

Appendix A. Supplementary data

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of epithelial structure is associated with human diseases such as inflammatory bowel disease and developmental diseases such microvillus inclusion disease.

In mice, much of intestinal development occurs during fetal life. Between embryonic day (E) 10.5 and E14.5, the midgut undergoes a dramatic elongation process, at which point the epithelium is pseudostratified (Grosse et al., 2011). Initiation of villus formation occurs around E14.5 (Walton et al., 2016), and intestinal crypts develop postnatally between P4 and P14 (Chin et al., 2017). In adults, the fully developed intestine consists of a single layer of columnar epithelial cells with mature crypts embedded in the lamina propria, and villi protruding into the intestinal lumen. At the base of the crypts are $Lgr5^+$ intestinal stem cells (ISCs), which divide and produce transit amplifying (TA) cells. TA cells rapidly proliferate and differentiate to give rise to secretory cells and absorptive enterocytes, which together constitute the epithelial cells of the villi.

The ISCs reside in an environment called the intestinal stem cell niche, where constitutive Wnt/β-catenin (canonical Wnt) signaling allows stem cells to maintain their undifferentiated and proliferative state (Wielenga et al., 1999; Staal et al., 2002), and recent findings have shown that essential Wnt signaling is provided by the surrounding mesenchymal cells (Aoki et al., 2016; Shoshkes-Carmel et al., 2018; Stzepourginski et al., 2017; Greicius et al., 2018; Degirmenci et al., 2018). FoxL1-expressing mesenchymal cells with telocyte properties ($FoxL1^+$ cells) are localized directly beneath the adult intestinal epithelium, separated from epithelial cells solely by the basement membrane (Aoki et al., 2016; Shoshkes-Carmel et al., 2018). Notably, ablation of *Porcupine* (a protein required for maturation of all Wnt proteins) in $FoxL1^+$ cells of adult mice causes complete cessation of canonical Wnt signaling to intestinal stem cells (Shoshkes-Carmel et al., 2018). This was an important discovery that showed that $FoxL1^+$ cells are critical for canonical Wnt signaling in the intestinal stem cell niche.

FoxL1 is a transcription factor most highly expressed in the gastric and intestinal mesoderm of mouse embryos (Kaestner et al., 1996), and ablation of this gene causes a delay in fetal intestinal development (Kaestner et al., 1997). FoxL1 transcription is regulated by binding of Gli, a major component of the Hedgehog signaling pathway, to *cis*-regulatory elements of FoxL1 (Madison et al., 2009; Coquenlorge et al., 2019). FoxL1 is initially expressed in mouse embryos at E8.5, where it is localized to mesenchymal cells surrounding the gut tube. Expression becomes restricted to the most subepithelial region of the lamina propria later in embryonic development (Kaestner et al., 1996). These early studies suggested that epithelial cells are involved in a cross-talk with the surrounding mesenchyme to regulate proliferation and differentiation during development.

$FoxL1^+$ cells in adult mice were found to express Wnt5a (Shoshkes-Carmel et al., 2018), which functions in short-range non-canonical Wnt signaling to neighboring cells. Unlike the canonical Wnt signaling pathway, non-canonical Wnt signaling is β-catenin-independent (Slusarski et al., 1997) and is important for establishing cell polarity (Qian et al., 2007; Gao et al., 2011; Gon et al., 2013). In vertebrates, Wnt5a is required for tissue elongation during organ development, as *Wnt5a*$^{-/-}$ (Wnt5a null) mice exhibit shortened tail, snout, limbs and bones (Yamaguchi et al., 1999). The role of Wnt5a during early intestinal development

has also been examined in Wnt5a null mice, which exhibit shortened intestines by E18.5 (Cervantes et al., 2009; Matsuyama et al., 2009; Wang et al., 2018). However, the cellular source of Wnt5a required for intestinal elongation has not been determined. Therefore, we derived *FoxL1-Cre; Wnt5a*$^{f/f}$ mice to elucidate the function of Wnt5a in FoxL1^{+} cells. We show that deletion of Wnt5a in FoxL1^{+} cells causes increased apoptosis in epithelial cells at E12.5, resulting in shortened intestines at birth. In contrast to some previously published studies, which suggested that apical-basal polarity is dysregulated in Wnt5a null mice, we found intact epithelial cell polarity in *FoxL1-Cre; Wnt5a*$^{f/f}$ mice. We conclude that FoxL1^{+} cells are a critical source of Wnt5a during midgut elongation. To our knowledge, this is the first study to characterize a specific mesenchymal cell population sustaining Wnt5a production during embryonic intestinal development.

2. Materials and methods

2.1. Mice

All procedures involving mice were conducted in accordance with approved Institutional Animal Care and Use Committee protocols. *FoxL1-Cre* mice were generated in the Kaestner Lab (Sackett et al., 2007), and *Wnt5a*$^{f/f}$ mice were procured from The Jackson Laboratory (*B6;129S-Wnt5atm1.1Krvl/J*) (Ryu et al., 2013). Genotyping of the two mouse lines were performed using the following primers: FoxL1-Cre F: GCG GCA TGG TGC AAG TTG AAT and R: CGC CGC ATA ACC AGT GAA ACA; Wnt5a F: GGT GAG GGA CTG GAA GTT GC and R: GGA GCA GAT GTT TAT TGC CTT C.

2.2. RNA isolation and qRT-PCR

To perform qRT-PCR, we first isolated RNA from dissected gut tubes. For P0 samples, we measured 4 cm of the small intestine proximal from the cecum, washed the tissue with cold 1× PBS, and homogenized the tissue in TRIzol reagent (Invitrogen). For E12.5 and E14.5 samples, we homogenized the whole gut in TRIzol. The homogenized tissues were either used immediately for RNA isolation or stored in −80 °C for later experiments. Total RNA was isolated using the RNeasy Mini kit for P0, and the RNeasy Micro kit for E12.5 and E14.5 (Qiagen), and cDNA was synthesized using Superscript II Reverse Transcriptase (ThermoFisher). qRT-PCR was performed using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix kit (Agilent Technologies). For quantifying Wnt5a gene expression, primer sets were designed flanking Exon 2 (F: GGT GCC ATG TCT TCC AAG TTC and R: CCG GAA CTG GTA CTG GCA TT). Actin primer sets were used for expression normalization (F: CCT TCT TGG GTA TGG AAT CCT G, and R: TGG CAT AGA GGT CTT TAC GGA), and relative expression was calculated using the $\Delta\Delta C_T$ method.

2.3. Tissue preparation for immunohistochemistry

E12.5, E14.5, and P0 tissue samples were prepared for paraffin-embedded immunohistochemical analyses. For E12.5, the embryos were euthanized by decapitation, briefly washed in cold 1× PBS, and fixed in 4% paraformaldehyde overnight in 4 °C. For E14.5 and P0 samples, intestines were dissected and washed briefly in cold 1× PBS, and fixed overnight in 4% paraformaldehyde in 4 °C. The next day, all tissues were rinsed in 1× PBS for 2 × 1 h, then dehydrated in 70% ethanol for 4 h, 95% ethanol for 2 × 3 h, and

100% ethanol for 2 × 1 h, and embedded in paraffin. Paraffin blocks were cut into 5 μm thick sections and were stored at room temperature until use.

2.4. Immunostaining and antibodies

Sections were deparaffinized in xylene and rehydrated in ethanol, and washed in 1× PBS for 5 min, followed by 1× PBS-T (0.25% Triton x-100) for 15 min, and 1× PBS for 3 × 5 min. Antigen retrieval was performed using a Tris-EDTA antigen retrieval buffer (pH 9.0). Sections were incubated for 30 min in Cas block buffer (Life Technologies) and incubated with primary antibodies overnight in 4 °C. The following day, the sections were washed 3 × 5 min. in 1× PBS, incubated with secondary antibodies for 2 h at room temperature, then washed and counterstained with Hoechst (Invitrogen) before imaging. The following antibodies were used for this study: FoxL1 (made in-house, 1:1500) E-cadherin (BD Bioscience, cat. 610,181, 1:300), EpCAM (Abcam, 1:300), Ezrin (Cell Signaling, 1:300), zona occludens 1 (ThermoFisher, 1:200), atypical Phosphokinase C (Santa Cruz Biotechnology, 1:200), Laminin (Abcam, 1:300), phospho-histone H3 (BMD millipore, 1:300), and Ki67 (BD Pharmagen, 1:300). All stained sections were imaged on a Leica TCS SP8 Confocal for immunofluorescence, and a Keyence BZ-X8100 microscope for brightfield imaging.

2.5. FoxL1 immunostaining

Immunohistochemical staining for FoxL1 was performed using the antibodies described previously (Aoki et al., 2016). Briefly, sections were deparaffinized in xylene and rehydrated in ethanol, and washed in 1× PBS for 5 min, followed by 1× PBS-T (0.25% Triton x-100) for 15 min. Antigen retrieval was performed using a Tris-EDTA antigen retrieval buffer (pH 9.0), quenched for 15 min with 3% hydrogen peroxide, blocked in Cas block buffer (Life Technologies) for 1 h, and incubated overnight with primary antibodies in 4 °C. The following day, the sections were incubated with Avidin and Biotin (Vector Labs) and incubated with biotinylated guinea pig IgG antibodies (Fisher Scientific, 1:300) for 30 min. Amplification was performed by incubation with streptavidin-peroxidase conjugate (Akoya) followed by incubation of the sections with Alexa 647 conjugated to streptavidin, and finally washed and counterstained with Hoechst (Invitrogen) before imaging.

2.6. Cell death detection

The apoptosis assay was performed using the ‘In Situ Cell Death Detection Kit’ (Millipore Sigma). Briefly, tissue sections were dewaxed using Xylene and ethanol gradients, and washed with 1× PBS. The sections were pre-treated with Proteinase K, washed with PBS, then incubated with TUNEL reaction mixture for 60 min in 37 °C. The slides were washed with PBS and counterstained with Hoechst before imaging as described above.

2.7. In situ hybridization

In situ hybridization on E12.5, E14.5, and P0 gut sections were performed using the RNAscope 2.5 LS Duplex Reagent Kit for the Leica Bond Rx (ACDBio), using commercially available Wnt5a and FoxL1 probes (ACDBio). The procedure was performed

by the Pathology Core at the Children's Hospital of Philadelphia using a Leica Bond Autostainer.

2.8. Statistical analysis

All statistical analyses were performed using the Prism software (GraphPad, version 8.2.1). Statistical significance was calculated using the Mann-Whitney test for comparing ranks.

3. Results

3.1. FoxL1 and Wnt5a are partially co-expressed in the developing intestine

Wnt5a is an essential developmental protein that is expressed in the intestinal mesenchyme from embryonic stages into adulthood (Lickert et al., 2001; Gregorieff et al., 2005). In the adult mouse intestine, Wnt5a has been shown to be expressed in FoxL1$^+$ cells, along with other key Wnt signaling ligands and receptors (Shoshkes-Carmel et al., 2018). However, up to now, the extent to which FoxL1 and Wnt5a are co-expressed during fetal development had not been characterized. Therefore, to determine whether FoxL1$^+$ cell-derived Wnt5a is essential for intestinal development, we first set out to assess co-expression of Wnt5a and FoxL1 in the midgut mesenchyme. We performed *in situ* hybridization for detection of FoxL1 and Wnt5a mRNAs in wildtype mice at E12.5, E14.5, and P0 (Fig. 1A–C). To validate nuclear localization of FoxL1 proteins in the subepithelial mesenchyme of the developing gut, we also performed immunofluorescent staining for FoxL1 (Fig. 1D–F). At all three developmental stages FoxL1 expression was restricted to the intestinal subepithelial regions, as previously reported (Kaestner et al., 1996; Aoki et al., 2016; Shoshkes-Carmel et al., 2018). On the other hand, Wnt5a was more broadly expressed in the intestinal mesenchyme at E12.5 and E14.5, and only became restricted to the subepithelium in later embryonic stages, which is evident at P0. Finally, to determine the function of Wnt5a produced by FoxL1$^+$ cells, we derived *FoxL1-Cre; Wnt5a$^{f/f}$* mice ('mutant' throughout) (Fig. 1G). This mouse model utilizes Cre-mediated deletion of exon 2 at the Wnt5a gene locus, resulting in an early truncation mutation (Ryu et al., 2013). We performed qRT-PCR to confirm reduced expression of Wnt5a in mutant mice compared to control mice (Fig. 1H). We did not expect to see complete ablation of Wnt5a expression in our model based on mRNA localization patterns, where Wnt5a is more broadly expressed than FoxL1 (Fig. 1A–C). However, because FoxL1$^+$ cells lie directly adjacent to the epithelium, these cells have the potential to play a more critical role in epithelial-mesenchymal crosstalk mediated by short-range Wnt signaling more than other Wnt5a-expressing cells. Therefore, we set out to further assess the functional role of FoxL1$^+$ cell-derived Wnt5a.

3.2. Foxl1-Cre; Wnt5a$^{f/f}$ mice have shorter intestines at birth compared to littermate controls

Wnt5a null mice present with severe developmental defects, including stunted skeleton, limbs, digits, and snout, demonstrating that Wnt5a is important for elongation of organs that require proximal to distal outgrowth (Yamaguchi et al., 1999). To assess the extent to which FoxL1$^+$ cell-derived Wnt5a recapitulates the null mouse phenotype, we measured body weight, body length, tail length, and hindlimb length of *FoxL1-Cre; Wnt5a$^{f/f}$* mice at birth (P0) (Fig. 2). While the mutants displayed a lower body weight compared to their littermate

controls (Fig. 2A), we did not detect stunted growth of body, tail, and hindlimb (Fig. 2B–D), confirming that our mutant mice had no developmental defects of non-intestinal organs. We hypothesized that the difference in weight was caused by intestinal defects in neonatal mutant mice. Therefore, we measured intestinal lengths of mice at P0 and found mutant small intestines to be significantly shorter than those of littermate controls (Fig. 2E,F). However, the shortened gut phenotype was not as severe as what has been reported in Wnt5a null mice (Cervantes et al., 2009). Nevertheless, our model showed that while $FoxL1^{+}$ cells are not the only source of Wnt5a in the intestinal mesenchyme, these cells produce Wnt5a that is necessary for proper midgut elongation during intestinal development.

3.3. Foxl1-Cre; $Wnt5a^{f/f}$ mice display normal epithelial architecture and apical-basal polarity

Wnt5a is a non-canonical Wnt ligand, and so its action is independent of β-catenin. In canonical Wnt signaling, its activation causes accumulation of β-catenin in the target cell's cytoplasm, which leads to its translocation into the nucleus. There, β-catenin acts as a signaling co-activator to TCF and LEF transcription factors to aid in transcription of cell cycle genes (Staal et al., 2002). Non-canonical Wnt signaling acts *via* several cytoplasmic effectors, where Wnt5a binding to specific receptors such as Ror2 (Oishi et al., 2003) and Frizzled-5 (Fzd5) (Ishikawa et al., 2001) activates the downstream effectors JNK, Rac1, and RhoA (Boutros et al., 1998). Although proteins such as c-Jun and NFAT are known to be involved in downstream transcriptional activation (Dejmek et al., 2006), non-canonical signaling involves signaling cascades that are non-transcriptional to regulate the planar cell polarity and apical-basal polarity pathways (Matsuyama et al., 2009; Gao et al., 2011). Cell polarity refers to a cell's ability to orient itself along the plane of the tissue. In the gut, apical-basal polarity pathways regulate the epithelial cells' ability to orient themselves between the apical (luminal) and basal sides, thus allowing the epithelium to endure the dynamic changes of the intestine during development while maintaining structural integrity. Previous studies have suggested that whole body Wnt5a deletion leads to abnormal epithelial structure during the pseudostratified epithelial stage, when the gut is elongating between E10.5 and E14.5 (Matsuyama et al., 2009). H&E staining of our mutant mice at E12.5, E14.5 and P0 showed no abnormal epithelial structure compared to control mice. (Fig. 3).

Next, we performed immunofluorescent staining to determine whether apical-basal polarity was dysregulated in our mice (Figs. 4, S1). Atypical phosphokinase C (aPKC), ZO-1, and Ezrin are all markers of apical polarity and are expressed exclusively on the luminal side of the intestinal epithelium. aPKC and ZO-1 are proteins involved in the formation of tight junctions (Giepmans and Moolenaar, 1998; Suzuki et al., 2001), and Ezrin is a protein that is required for formation of microvilli during development (Vaheri et al., 1997). By visualizing localization of these three proteins by immunofluorescent staining, we could assess apical-basal polarity via three structural components that define epithelial structural integrity (Figs. 4A–F, S1A–L). Furthermore, we assessed localization of laminin, which is a basal membrane protein marker involved in the establishment of apical-basal polarity (Fig. 4G–L) (O'Brien et al., 2001; Cervantes et al., 2009). However, we did not observe any dysregulation of these apical and basal polarity proteins at E12.5, E14.5, and P0.

Therefore, we conclude that the short-gut phenotype in *FoxL1-Cre; Wnt5a*$^{f/f}$ mice is not due to dysregulation of epithelial cell polarity.

3.4. Foxl1-Cre; Wnt5a$^{f/f}$ mice have normal epithelial proliferation but have increased apoptosis at E12.5

To identify the mechanisms by which Wnt5a deletion in FoxL1^{+} cells causes gut shortening, we next assessed the rate of proliferation of intestinal epithelial cells, which rapidly proliferate during midgut elongation. Immunofluorescent staining for Ki67 at E12.5 and P0 showed that the epithelial cells of Wnt5a deleted mice do not have decreased proliferation (Fig. S2). Because Ki67 is present in cells that are in any active phase of the cell cycle, it is challenging to quantify rate of proliferation at E12.5 using the Ki67 marker. Therefore, we stained for phosphorylated Histone H3 (pHistone H3), which only detects mitotically active cells (Fig. 5) (Kim et al., 2017). For E12.5 intestines, we quantified the relative rate of proliferation by counting the number of pHistone H3-positive cells in the epithelium, divided by the area of the epithelium (in pixels) in the image (Fig. 5C). For P0 samples, we quantified the rate of proliferation by dividing the total number of pHistone H3-positive cells by the number of intervillus regions in the image (Fig. 5F). Using this method, we found no significant changes in the rate of proliferation between Wnt5a deleted mice and control mice at E12.5 and P0. This was an expected finding for mice at E12.5, as previous studies have shown that Wnt5a and non-canonical signaling do not affect proliferation during early embryonic stages (Wang et al., 2018). However, the role of Wnt5a in regulating proliferation in neonatal mice was not known up to this point, as all previous Wnt5a functional studies in the intestine used Wnt5a null mice, which are perinatal lethal (Yamaguchi et al., 1999). Our assessment reveals that Wnt5a deletion from FoxL1^{+} cells does not affect epithelial proliferation at birth, a critical time when the gut is priming for postnatal crypt development.

Finally, in Wnt5a null mice, gut shortening has been shown to be caused by an increase in apoptotic activity in epithelial cells, leading to increased shedding of apoptotic cells into the intestinal lumen (Wang et al., 2018). To determine whether gut shortening in our model is caused by increased apoptotic activity, we performed *in situ* cell death detection by TUNEL staining, which labels double strand breaks in the DNA (Kyrylkova et al., 2012). We determined the rate of apoptosis in the epithelium using the same quantification methods described in Section 3.4, and detected a significant increase in epithelial cell apoptosis in mutant mice at E12.5 (Fig. 6A–C). Rates of apoptosis at P0 on the other hand were indistinguishable between the two groups (Fig. 6D–F). In sum, we conclude that Wnt5a production by FoxL1^{+} cells is required for midgut elongation, and loss of Wnt5a production by these cells causes a shortened intestine by increasing epithelial apoptosis.

4. Discussion

Our study shows that when Wnt5a is ablated from FoxL1-expressing mesenchymal cells, epithelial cells undergo increased apoptotic activity during midgut elongation, resulting in shorter intestines at birth. To the best of our knowledge, this is the first study to successfully identify a specific mesenchymal cell population to be an important source of Wnt5a during embryonic gut development. In addition to the Wnt5a null model, previous studies utilized

more broadly expressed markers for Wnt5a ablation in the intestine such as ubiquitously expressed Cag-Cre (Miyoshi et al., 2012), or modeled gut shortening phenotype by ablating the Wnt5a receptor Ror2 (Yamada et al., 2010), and its antagonists Sfrp1 and GPC6 (Matsuyama et al., 2009; Shi et al., 2020). While it has been known for some time that Wnt5a is produced in the intestinal mesenchyme, we showed that deleting Wnt5a from FoxL1^{+} subepithelial cells leads to an alleviated form of the Wnt5a null phenotype.

Our experiments in Fig. 1 show that while Wnt5a and FoxL1 are co-localized in the mesenchymal cells directly beneath the epithelium, during early intestinal development Wnt5a is more broadly expressed in the mesenchyme than FoxL1. With this knowledge, we expected that the gut shortening phenotype would be milder than what is observed Wnt5a null mice. In fact, Wnt5a null mice have almost an 80% reduction of small intestinal length compared to wildtype gut (Yamaguchi et al., 1999), while *FoxL1-Cre; Wnt5a$^{f/f}$* mice display only a 15% reduction in length (Fig. 2E–G). Furthermore, the body, tail, and limbs of Wnt5a null mice are significantly truncated at E18.5, which demonstrates the importance of Wnt5a in the development of numerous organs (Yamaguchi et al., 1999). By contrast, we did not observe any changes in body, tail, and hindlimb lengths in the *FoxL1-Cre; Wnt5a$^{f/f}$* mice (Fig. 2B–D). This raises concerns about potential consequences of global null models when studying gene function. In this particular case, we are left to wonder whether the significant shortening of the intestines in Wnt5a null mice is largely contributed by the *overall* stunted growth of the animal. A future functional study using a Wnt5a deletion model that only targets the intestinal mesenchyme, such as with a PDGFRα-Cre driver, may be useful to uncover additional functions for Wnt5a in gut development.

In early embryonic stages, Wnt5a is required to establish planar cell polarity by polarization of node cells for breaking of left-right symmetry (Mineshigi et al., 2017), while in intestinal development Wnt5a is associated with apical-basal polarity. However, studies have presented conflicting conclusions with respect to whether apical-basal polarity is dysregulated in Wnt5a null mice, where some claim that Wnt5a is regulates apical-basal polarity protein localization and is thus is required for cell polarity establishment in the intestine (Matsuyama et al., 2009), while others claim that Wnt5a is not involved in this process (Cervantes et al., 2009; Wang et al., 2018). The disparities in the role of Wnt5a in establishing cell polarity in the intestine reflect the challenges of studying non-canonical Wnt signaling pathways, which involve numerous ligands, receptors, and downstream effector proteins. Furthermore, the mechanism of protein rearrangements in the cytoplasm required for establishing cell polarity are largely non-transcriptional and therefore remain elusive. Yet, with the acknowledgement that the *FoxL1-Cre; Wnt5a$^{f/f}$* mouse model ablates Wnt5a only from a subset of mesenchymal cells during development, our findings support previous observations that Wnt5a *does not* regulate apical-basal cell polarity, as evidenced by normal epithelial structural integrity and polarity protein localization at E12.5, E14.5, and P0 (Figs. 3, 4, S1).

In conclusion, we have identified FoxL1^{+} mesenchymal cells as a source of Wnt5a during midgut elongation, where we observed shortened intestines in *FoxL1-Cre; Wnt5a$^{f/f}$* mice compared to littermate controls. The increase in apoptosis observed in the mutant mice suggests that cause of intestinal shortening is due to increased epithelial cell death during

midgut elongation and is independent of apical-basal polarity protein localization. This is the first study to identify a mesenchymal cell type contributing to the secretion of Wnt5a from the mesenchyme to regulate intestinal development. With our findings we hope emphasize the importance of performing functional studies using cell-type specific gene ablation models to eliminate confounding effects of global gene deletion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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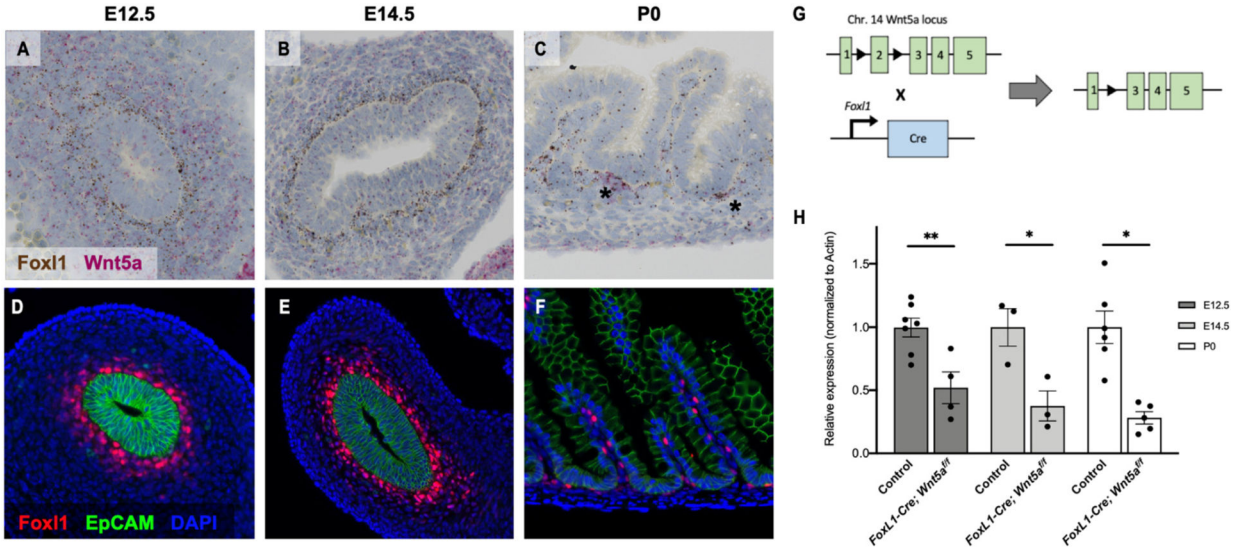

Fig. 1.

Validation of Wnt5a and FoxL1 co-localization in embryonic and neonatal mouse intestines. (A–C) *In situ* hybridization of *FoxL1* and *Wnt5a* in wildtype mice using RNAscope shows FoxL1 mRNA localized to the subepithelium, while *Wnt5a* is expressed more broadly in the intestinal mesenchyme at E12.5 and E14.5. At P0, Wnt5a becomes localized to the subepithelial intervillus regions (asterisks). (D–F) Immunohistochemical staining for FoxL1 shows nuclear expression in subepithelial regions of the intestine at E12.5, E14.5, and P0. (G) Schematic for the *FoxL1-Cre; Wnt5a*$^{f/f}$ mouse model. (H) Quantification of *Wnt5a* mRNA levels in *FoxL1-Cre; Wnt5a*$^{f/f}$ and control mice shows decreased expression in experimental mice. (*) indicates p value <0.05, (**) indicates p-value <0.01.

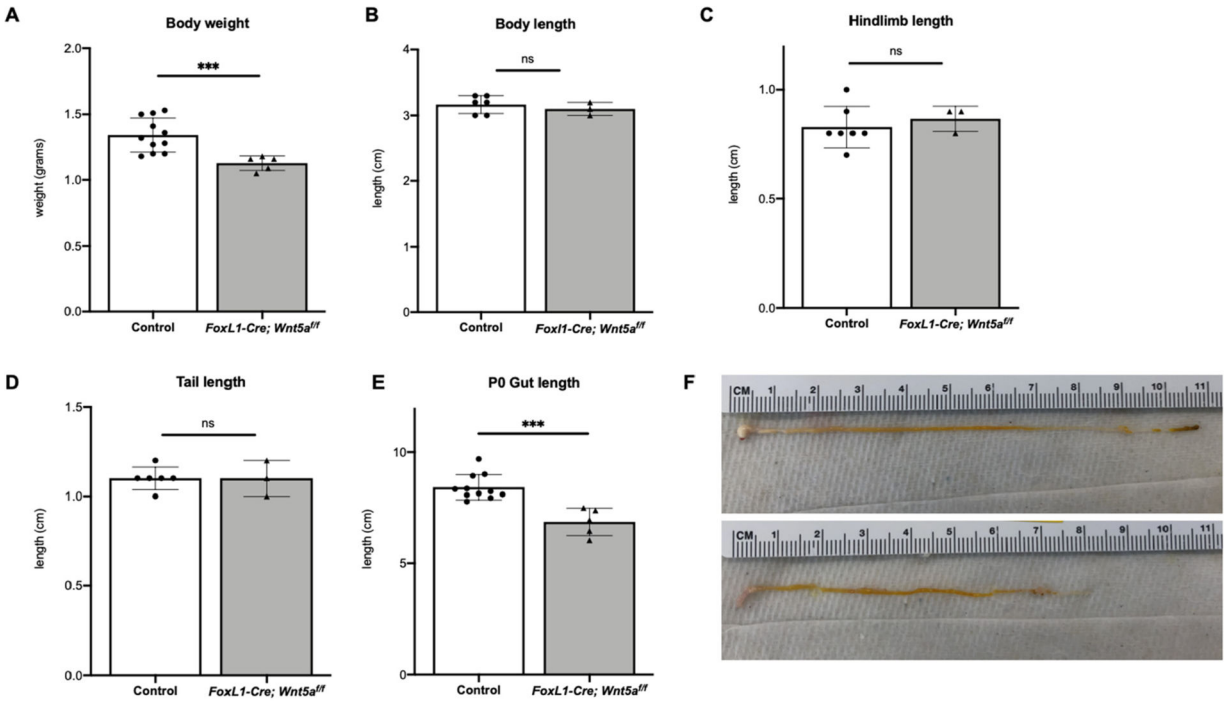

Fig. 2.

Physical assessment of mutant mice at P0. (A) Compared to control mice, mutant mice have reduced body weight at birth, but normal (B) body length, (C) hindlimb length, and (D) tail length. (E) Quantification of gut length in mutant and control mice at P0. (F) Representative images of control (top) and mutant (bottom) mouse intestines at P0. (***) indicates p-value <0.001, (ns) indicates no significance.

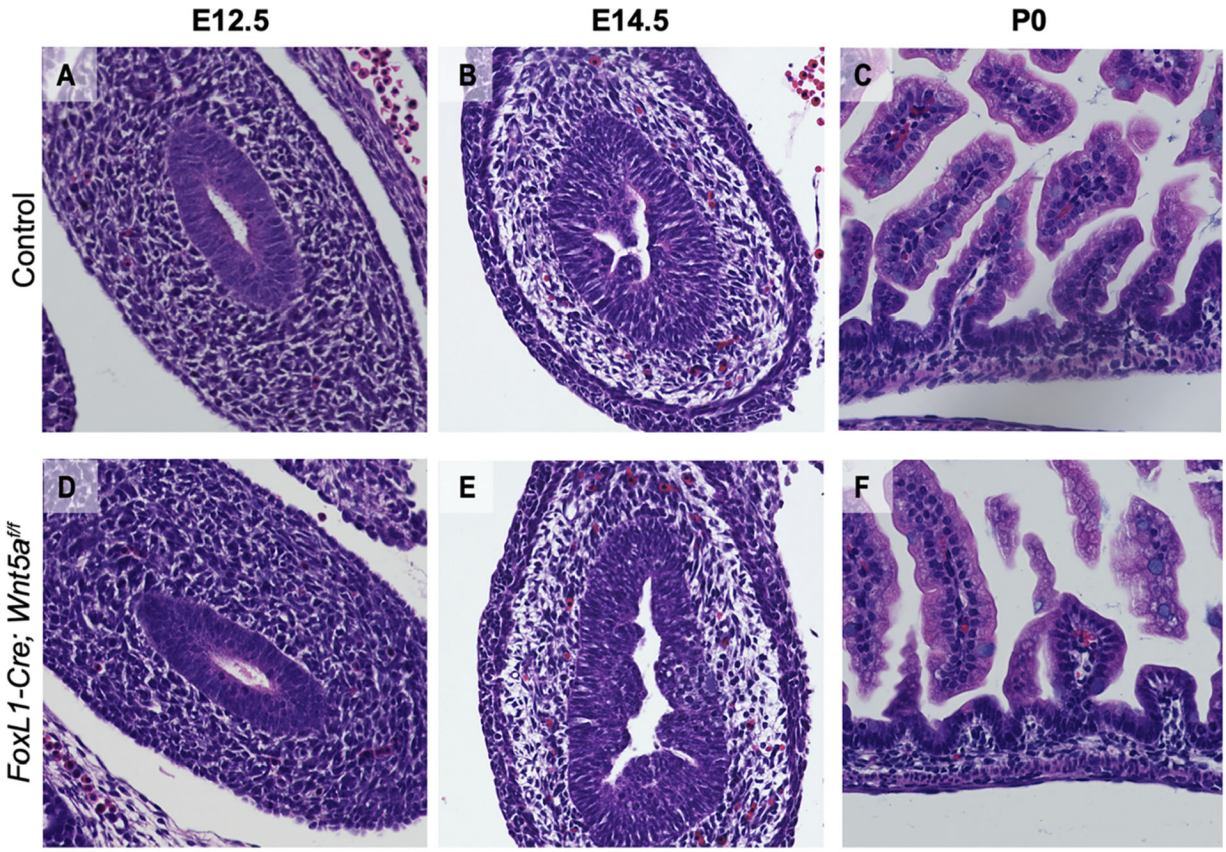

Fig. 3.

H&E images of the developing gut. *FoxL1-Cre; Wnt5a*$^{f/f}$ mice have epithelial architecture comparable to the controls at (A,D) E12.5, and (B,E) E14.5, and (C, F) P0.

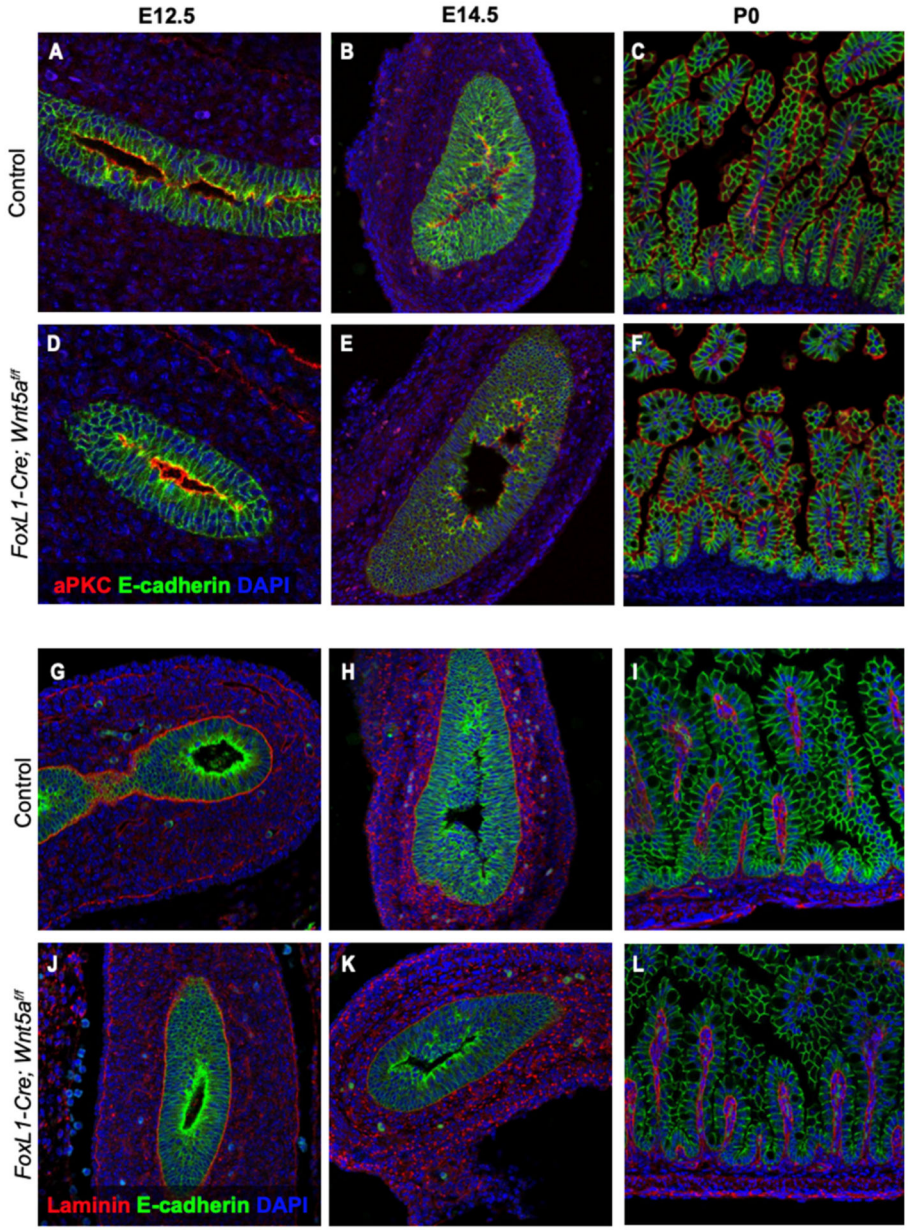

Fig. 4.

Immunofluorescent staining for polarity proteins at E12.5, E14.5, and P0. (A–F) aPKC is an apical polarity protein and marks the luminal side of the epithelium. (G–L) Laminin is localized to the basal side of the intestinal epithelium.

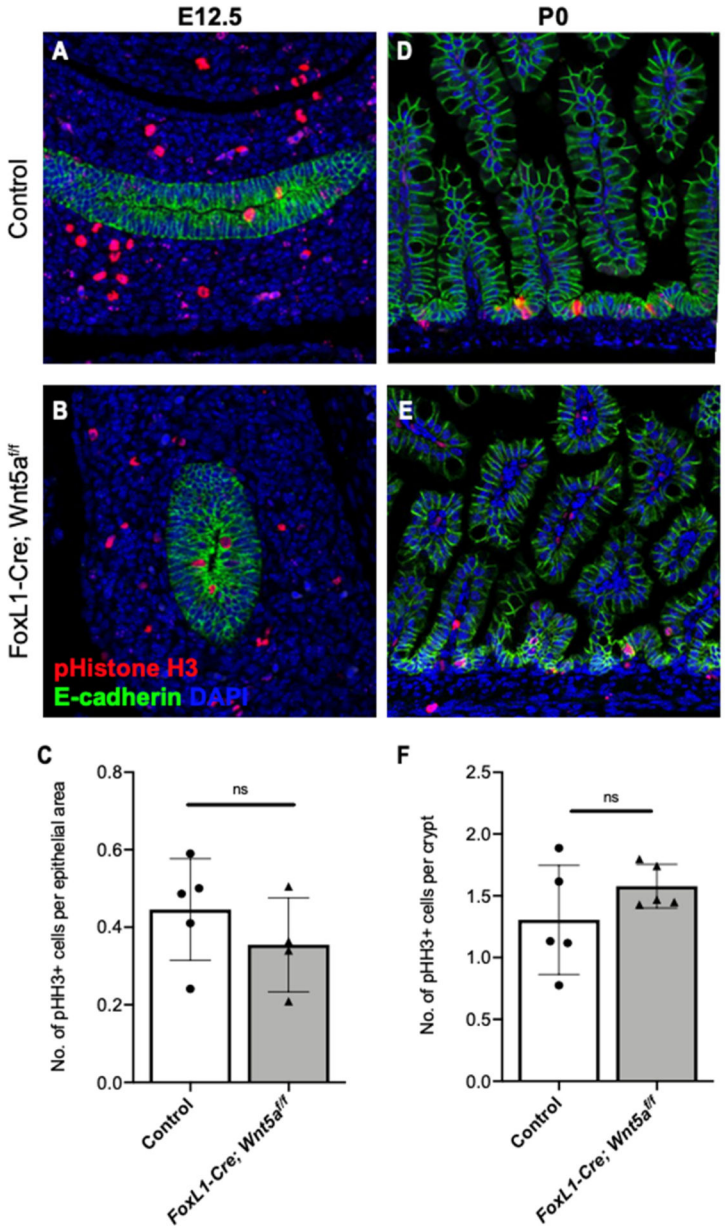

Fig. 5.

Assessment of proliferation in *FoxL1-Cre; Wnt5a*$^{f/f}$ mice. (A,B) Immunofluorescent staining for control and experimental mice at E12.5. (C) Rate of proliferation in E12.5 intestines quantified by density (number of phosphorylated histone H3 (pHistone H3) positive cells divided by area of epithelium). For each embryo, four different regions of the intestine were imaged and quantified. (D, E) Immunofluorescent staining for control and mutant mice at P0. (F) Rate of proliferation in P0 intestines quantified by number of phosphorylated histone H3 (pHistone H3) positive cells in the epithelium per length of intestine. For each neonatal sample, 6–8 different regions of the intestine were imaged and quantified. (ns) indicates no statistical significance.

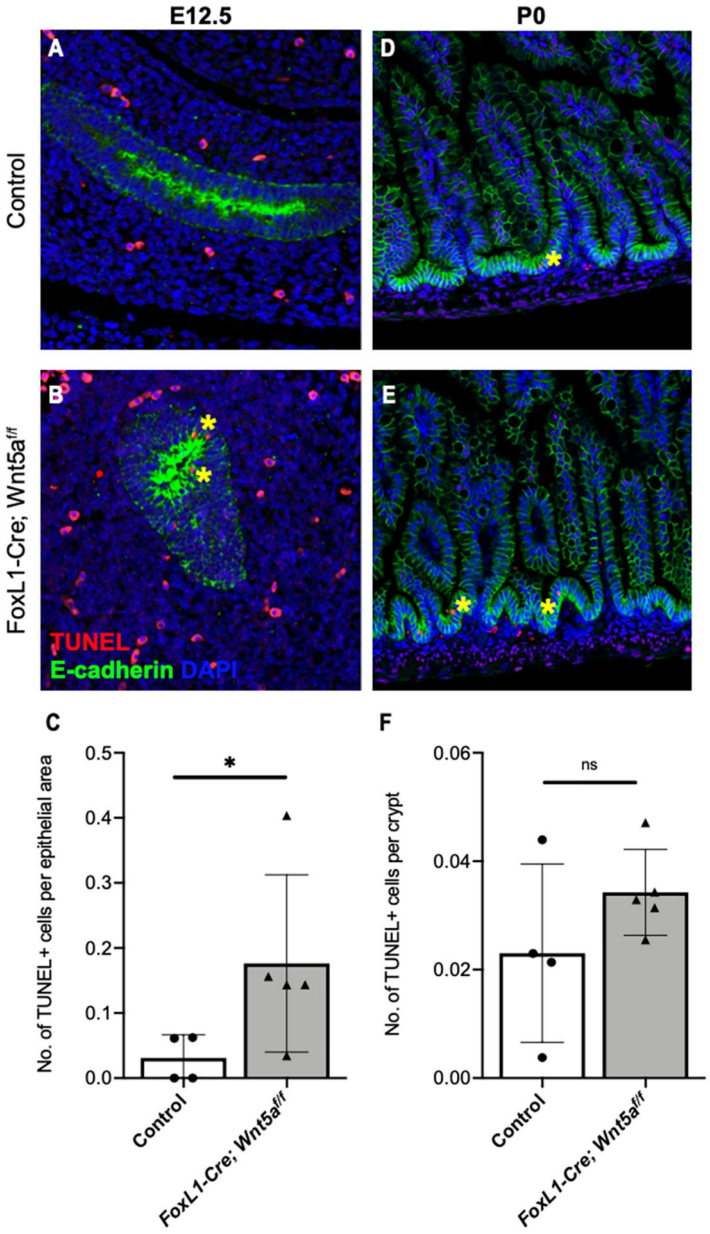

Fig. 6.

Quantification of apoptosis by TUNEL staining. (A,B) Immunofluorescent staining image for control and mutant mice at E12.5. (C) Rate of apoptosis in E12.5 intestines quantified by density (number of TUNEL-positive cells divided by area of epithelium). For each embryo, four different regions of the intestine were imaged and quantified. (D,E) Immunofluorescent staining for control and mutant mice at P0. (F) Rate of apoptosis in P0 intestines quantified by number of phosphorylated histone H3 (pHistone H3) positive cells in the epithelium divided by length of intestine. For each neonatal sample, 6–8 different regions of the intestine were imaged and quantified. (*) indicates significant at $p < 0.05$, (ns) indicates no significance.