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ORIGINAL RESEARCH

The Endosomal Protein Endotubin Is Required for Enterocyte Differentiation

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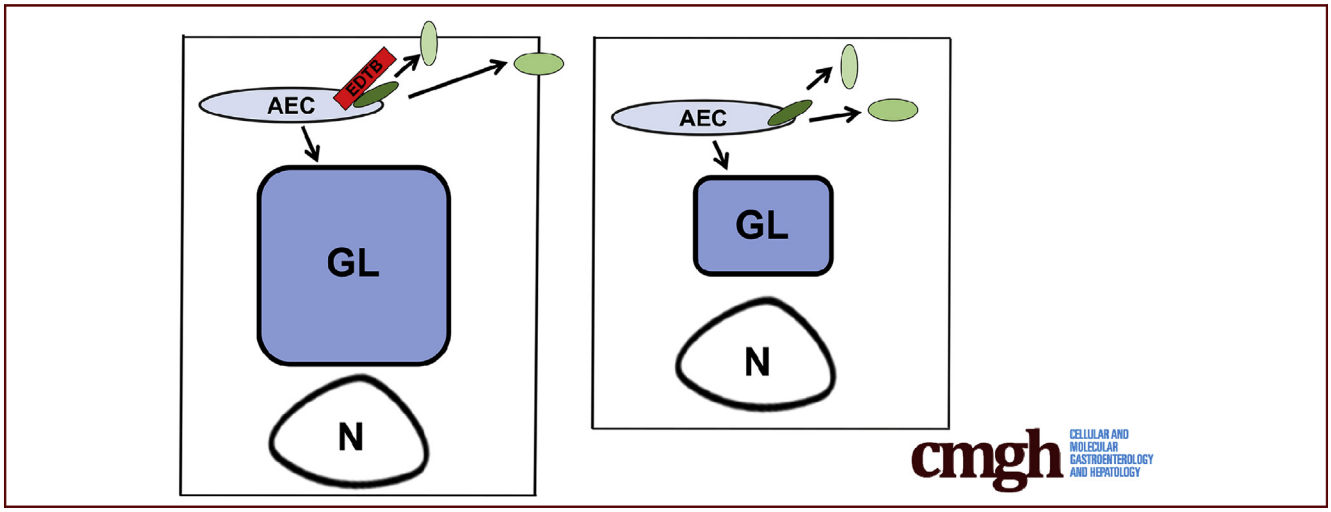

SUMMARY

The apical endosomal protein endotubin is highly expressed during enterocyte development. Here, we show that endotubin is required for membrane trafficking and enterocyte morphogenesis. We propose that endotubin serves as a scaffold that modulates apical membrane traffic.

BACKGROUND & AIMS: During late embryonic development and through weaning, enterocytes of the ileum are highly endocytic. Defects in endocytosis and trafficking are implicated in neonatal disease, however, the mechanisms regulating trafficking during the developmental period are incompletely understood. The apical endosomal protein endotubin (EDTB) is highly expressed in the late embryonic and neonatal ileum. In epithelial cells in vitro, EDTB regulates both trafficking of tight junction proteins and proliferation through modulation of YAP activity. However, EDTB function during the endocytic stage of development of the intestine is unknown.

METHODS: By using Villin-CreERT2, we induced knockout of EDTB during late gestation and analyzed the impact on endocytic compartments and enterocyte structure in neonates using immunofluorescence, immunocytochemistry, and transmission electron microscopy.

RESULTS: Deletion of the apical endosomal protein EDTB in the small intestine during development impairs enterocyte morphogenesis, including loss of the apical endocytic complex, defective formation of the lysosomal compartment, and some cells had large microvillus-rich inclusions similar to those observed in microvillus inclusion disease. There also was a decrease in apical endocytosis and mislocalization of proteins involved in apical trafficking.

CONCLUSIONS: Our results show that EDTB-mediated trafficking within the epithelial cells of the developing ileum is important for maintenance of endocytic compartments and enterocyte integrity during early stages of gut development. *(Cell Mol Gastroenterol Hepatol 2018;5:145–156; https://doi.org/10.1016/j.jcmgh.2017.11.001)*

Keywords: Trafficking; Endotubin; Rab; Tight Junction; Endosomes.

Abbreviations used in this paper: **AEC, apical endocytic complex; AP, alkaline phosphatase; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/cas9 endonuclease; EDTB, endotubin; EEA1, early endosomal antigen 1; G, guide; GFP, green fluorescent protein; GTPase, guanosine triphosphatase; KO, knockout; LAMP1, lysosome-associated membrane protein 1; MAMDC4, MAM domain containing 4; MVID, microvillus inclusion disease; P, postnatal day; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TBST, tris-buffered saline with 0.1% tween-20; TEM, transmission electron microscopic; TJ, tight junction.**

Most current article

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The intestinal epithelium serves as a selective barrier to macromolecules and pathogens, and dysfunction of the enterocytes can lead to disease in both neonates and adults.[1–8] During early development of the ileum, the enterocytes of the villus undergo a stage in which they are highly endocytic, with a well-developed tubular apical endosomal complex and extensive lysosomal system.[9,10] In rodents, the apical endocytic complex (AEC) forms late in gestation and is present in the apical cytoplasm through weaning.[9–13] These membranes consist of both tubular and vesicular endosomes adjacent to the apical plasma membrane, with the giant lysosome filling the majority of the apical cytoplasm. This endocytic complex may provide nutrition through the uptake of macromolecules from the lumen and also facilitates the transfer of growth factors such as nerve and epidermal growth factors from the lumen to the circulation.[13–15] Both the AEC and giant lysosome are lost at the time of weaning.

The importance of vesicular trafficking in enterocyte development has been established in studies with knockout of the small guanosine triphosphatases (GTPases) Rab8a and Rab11a, which result in defective localization of apical enzymes and a poorly developed microvillus border.[16–19] Studies with knockout of the transcription factor Cdx2 showed that Cdx2 regulates a network of genes that are involved in endosome/lysosome trafficking.[20] Finally, microvillus inclusion disease (MVID) has been shown to be caused by a mutation in myosin Vb or syntaxin 3, proteins known to mediate apical trafficking, and loss of the actin nucleator Arp2/3 results in defects in endolysosomal assembly and vesicle trafficking.[2,21–25] Endotubin (EDTB) also known as MAM domain containing 4 (MAMDC4) is an integral membrane protein that is concentrated in the tubular apical endosomes of developing intestine.[11,12] In the rodent, EDTB begins to be expressed late in embryonic development and retains high expression levels until weaning.[11] It still is expressed in enterocytes after weaning, but at approximately one half the levels found during development. Despite its endosomal location, experiments in epithelial cells in vitro have shown that EDTB regulates the assembly and maintenance of tight junctions (TJs) and interacts with TJ proteins and transcriptional co-factors.[26,27] However, the role of EDTB in the regulation of membrane trafficking and function in the developing intestine is unknown.

To better understand the importance of apical membrane trafficking during intestinal development, we used inducible intestinal epithelial knockout of EDTB. EDTB was knocked out late in gestation before assembly of the AEC and was evaluated shortly after birth. We found that loss of EDTB resulted in disruption of the AEC and the giant lysosome. Furthermore, we observed loss of some TJ proteins from the lateral membranes and the occasional formation of large microvillus-containing inclusions. These results implicate EDTB as an essential regulator of membrane trafficking during intestinal development.

Materials and Methods

Mice

The EDTB (MAMDC4) targeting vector was generated containing a neomycin selectable marker and loxP recombination sites flanking exons 2 and 10. Deletion of exons 2 through 10 results in a frame shift mutation and premature stop codon. The mouse colony containing MAMDC4$^{fl/+}$ and MAMDC4$^{fl/fl}$ offspring was generated and maintained by the Experimental Mouse Shared Resource (EMSR) at the University of Arizona Cancer Center and supported by the National Cancer Institute of the National Institutes of Health P30 CA023074. Villin-CreErt2 and MAMDC4$^{fl/fl}$ animals were crossed to generate MAMDC4$^{fl/+}$/Villin-CreErt2 males. MAMDC4$^{fl/+}$/Villin-CreErt2 males then were mated with MAMDC4$^{fl/fl}$ females. Pregnant females were subjected to intraperitoneal injections with 100 uL of 10 mg/mL 4-hydroxytamoxifen (cat. T176-50; Sigma, St. Louis, MO) on day 15 of gestation to induce expression of Cre recombinase. Offspring were collected at postnatal day 3 (P3).

Genotyping

Tail clips were obtained and digested in 90 μL tail digestion buffer (100 mmol/L Tris, pH 8.0, 0.5 mmol/L EDTA, 0.2% sodium dodecyl sulfate [SDS], 200 mmol/L NaCl, 100 μg proteinase K) for 1 hour at 55°C. After digestion, 300 μL H_2O was added, samples were vortexed, and then incubated at 95°C for 15 minutes. Debris was removed by centrifugation and 2 μL of supernatant was used for polymerase chain reaction (PCR) reactions using TopTaq Master Mix Kit (cat. 200403; Qiagen).

The primers used were as follows: mouse EDTB neo forward, GGGGTTTGCTCGACATTG; mouse EDTB wild type reverse, ATACAGCTTTGATGGGGCTTC; mouse EDTB wild type forward, GTGGCGGTTCTTGGTATATGTC; villin-cre forward, CAAGCCTGGCTCGACGGCC; and villin-cre reverse, CGCGAACATCTTCAGGTTCT.

Tissue Preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Animals were euthanized and the abdominal wall was incised. The intestine was exposed and the ileum was flushed with ice-cold phosphate-buffered saline (PBS) and divided into proximal and distal segments. Tissue was fixed in 4% paraformaldehyde in PBS for 4 hours at room temperature for paraffin embedding. After fixation, tissue was washed within PBS and dehydrated through an ethanol series (2 × 30 min in 50%, 70%, and 100%) followed by 50:50 ETOH:xylene and 100% xylene. Tissue was transferred to paraffin at 60°C for 1 hour and to fresh paraffin overnight at 60°C. For electron microscopy, ileum was fixed in 2% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4). Postfixation was performed in aqueous 1% OsO_4 for 2 hours at 4°C, and en bloc stained with 0.5% uranyl acetate, followed by dehydration in ethanol and embedding in Spurrs resin (Electron Microscopy Sciences, Hatfield, PA). Sections