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Retinaldehyde dehydrogenase 2 is down regulated during duodenal atresia formation in *Fgfr2IIIb −/−* mice

Peter F. Nichol, MD/PhD[Assistant Professor],
Section of Pediatric Surgery, Department of Surgery, University of Wisconsin SMPH, Madison, WI

John D. Tyrrell, BS, and
University of Utah, Salt Lake City, UT

Yukio Saijoh, PhD[Assistant Professor]
Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT

Abstract

Background—Homozygous null mutation of *Fgfr2IIIb* or its ligand *Fgf10* results in duodenal atresia in mice. Mutations of either of these genes in humans cause Matthew-Wood syndrome and associated duodenal stenosis. Recently, mutations in the retinol-binding protein receptor gene *STRA6* were reported to be implicated in this syndrome as well. This suggests that the retinoic acid (RA) signaling pathway interacts with the Fgf10-Fgfr2IIIb signaling pathway during duodenal development. Accordingly, we hypothesized that *Fgfr2IIIb−/−* mouse embryos would exhibit disruptions in expression of *Raldh2,* the gene for the enzyme that regulates the final step in the conversion of vitamin A to the active form RA, during duodenal atresia formation.

Materials and Methods—*Fgfr2IIIb −/−* mice were generated from heterozygous breedings. Embryos were harvested between embryonic day (E) 11.0 to E13.5 and genotyped by PCR. Duodenums were dissected out, fixed and photographed. Whole mount and section in situs were performed for *Raldh2.*

Results—*Fgfr2IIIb−/−* embryos demonstrate subtle changes in the duodenal morphology by E11.5 with complete involution of the atretic precursor by E13.5. *Raldh2* appears to be down regulated as early at E11.5 in the atretic precursor a full 2 days before this segment disappears.

Conclusions—In *Fgfr2IIIb−/−* mouse embryos, a reduction of *Raldh2* expression is observed within the region that is forming the atresia. This is the first demonstration of such an event in this model. As in humans, these results implicate disruptions between Fgfr2IIIb receptor function and RA signaling in the formation of this defect and indicate that *Fgfr2IIIb−/−* mouse embryos are a valid model for the study of the atretic spectrum of defects in human duodenal development.

Keywords

Duodenal atresia; mouse; Fgfr2IIIb; Raldh2; retinoic acid; down regulation; gene expression; Matthew-Wood syndrome

Corresponding Author: Peter F. Nichol MD/PhD, Assistant Professor, Section of Pediatric Surgery, Department of Surgery, University of Wisconsin SMPH, Madison, WI, nichol@surgery.wisc.edu, (O) (608) 263-9419, (F) (608) 261-1876.

Introduction

During duodenal development in humans, the lumen of the proximal duodenum becomes occluded by an endodermal plug at Carnegie Stage (CS) 17 and then begins to recanalize two days later as CS 18. The first description of these events was published by Tandler in 1900 and he went on to hypothesize that a failure of the recanalization step could result in duodenal atresia (1). Recently a genetic animal model for duodenal atresia has been described in which the defect arises from a deletion of the *IIIb* exon of the *Fibroblast growth factor receptor 2* gene (*Fgfr2*). This results in the loss of the Fgfr2IIIb isoform of the receptor throughout the embryo during development (2). The mutant embryos form atresias in the second portion of the duodenum similar to humans. The legitimacy of this model has been bolstered by the observation that mutations in the *Fibroblast Growth Factor Receptor 2* gene or the gene encoding its ligand *Fibroblast Growth Factor 10* result in duodenal stenosis in humans (3). Interestingly, the morphogenesis of atresia in the animal model has not been described and signaling pathways downstream of Fgfr2IIIb have not been identified in the formation of this defect.

Disruptions in the retinoic acid (RA) signaling pathway have been implicated in the formation of duodenal stenosis, a type of atretic defect in humans (4). The RA signaling pathway interacts with Fgfr2IIIb signaling during lung and pancreas development in mice (5, 6). Loss of *Raldh2* expression (a gene that encodes the enzyme that regulates the final step in the conversion of vitamin A to RA) impedes the generation of Fgf10 in the lung, thus disrupting lung development (5). In the pancreas, loss of *Raldh2* expression results in agenesis of the dorsal pancreatic bud (6). Based on this evidence, we hypothesized that Fgfr2IIIb signaling would interact with the retinoic acid signaling pathway and we specifically predicted that loss of Fgfr2IIIb function would down regulate *Raldh2* expression in the duodenum during atresia formation in *Fgfr2IIIb−/−* mouse embryos.

Materials and Methods

Animals

IACUC approval for these studies was obtained from the University of Wisconsin School of Medicine and Public health (P.F.N. protocol # M02258). All animals were maintained in a clean facility with access to fresh food and water and kept on a 12 hour alternating light/dark cycle. *Fgfr2IIIb−/−* embryos (7) were generated through traditional heterozygous *Fgfr2IIIb +/−* breedings. Forty percent of the embryos developed a duodenal atresia as has been previously reported (2). Litters were harvested between E11.0 and E13.5 into cold PBS. Genotyping was performed on yolk sacs as described previously (7). Embryos were fixed overnight in 4% paraformadehyde (PFA) at 4°C, and intestines were dissected the following day under a stereoscopic microscope and photographed.

Whole mount in situ hybridization

Samples were dehydrated through a series of escalating PBS-Tween methanol steps then stored overnight at −20 °C. The following day they were rehydrated into PBS-Tween, treated with hydrogen peroxide and proteinase K, and in situ hybridization was performed at 70 °C for *Raldh2* with antisense probes (8). Specimens were stained at 37 °C, washed in PBS-Tween and fixed with 4% PFA. Photographs were taken under a dissecting light microscope.

Section in situ hybridization

Embryos were harvested into cold phosphate buffered saline (PBS) at E 11.5 and fixed overnight in Bouin's fixative at 4°C. They were dehydrated through a series of escalating

PBS/Ethanol steps and embedded into Paraffin. Sections were taken a 10 micrometer thickness and floated onto slides. Sections were de-waxed and rehydrated. Slides were treated proteinase K followed by Acetic anhydride and triethanolamine, re-fixed with paraformadehyde, and section in situs were performed at 60 °C with antisense probes for *Raldh2*. Sections were stained overnight at 37°C, fixed with 4% PFA and cover slipped with glycerol. Photographs were taken under a light microscope.

Results

Morphogenesis of duodenal atresia in *Fgfr2IIIb−/−* mouse embryos

The timing of duodenal atresia in the *Fgfr2IIIb−/−* mouse model has not been previously described. The atresia forms in the second portion of the duodenum (2). We examined this process in early development with standard stereoscopic microscopy on partially dissected whole mount specimens. We observed that the proximal duodenum of in *Fgfr2IIIb−/−* embryos appeared slightly narrowed at E11.5 (Figure 1B, black arrow) compared to the control (Figure 1A). This is the equivalent of Carnegie stage (CS) 16 in a human (the stage just before the endodermal plug forms). By E12.5 (CS 18- when the plug first begins to recanalize in humans), the midpoint of the proximal limb of the duodenum had narrowed more significantly (Figure 1D, black arrow) in comparison to the control (Figure 1C). By E13.5, or CS 20, the mid-portion of the proximal duodenum was absent (Figure 1F, black arrow) indicating that the atresia had completely formed. The results suggest that the timing of the changes to the duodenum leading up to atresia formation occur rather rapidly, beginning at the equivalent of CS 16 in the human and reaching completion around CS 20.

Expression of *Raldh2* in the developing intestine and duodenum

Disruptions in retinoic acid signaling have been linked to duodenal stenosis in humans. We hypothesized that similar disruptions would occur during duodenal atresia formation in mice. Prior to testing this, we first characterized the expression pattern of *Raldh2* in normal mouse duodenum by whole mount in situ hybridization. We observed that at E11.0 *Raldh2* was robustly expressed in the proximal intestine with diminishing intensity distally and at this stage was altogether absent in the early cecum and colon (Figure 2A). To determine the tissue specific expression within the intestine we performed a section in situ hybridization at E11.5. This demonstrated expression of *Radh2* within the duodenum was limited to the mesoderm and that the expression was of equal intensity both in the proximal and distal duodenum (Figure 2B).

Expression of *Raldh2* during duodenal atresia formation

To determine whether *Raldh2* expression was altered during duodenal atresia formation, we examined *Raldh2* expression in *Fgfr2IIIb−/−* embryos between E11.5 and E12.5 by in situ hybridization. We observed that at E11.5 there appeared to less *Raldh2* expression in the proximal duodenum (Figure 3B, black arrowheads) in comparison to the distal duodenum (Figure 3B, white arrowheads) of *Fgfr2IIIb−/−* embryos. Furthermore, as in Figure 1B (Black arrow), the proximal duodenum was beginning to exhibit evidence of narrowing (Figure 3B).

By E12.5, the reduced intensity of *Raldh2* expression was very focal within the mid-proximal duodenum (Figure 4B, black arrowhead in proximal duodenum) where the atresia was forming. Narrowing of the duodenum within this region was also more evident than at the previous stage (Compare Figure 3B, to 4B). In the *Fgfr2IIIb−/−* embryo that hadn't formed an atresia (Figure 4C), *Raldh2* expression was uniform throughout the duodenum, but reduced compared to the control (Figure 4A). In fact, the overall expression pattern of *Raldh2* in the proximal intestine appeared to be reduced in both *Fgfr2IIIb−/−* embryos

(Figure 4B and C). It appeared that *Raldh2* expression in the mutants was restricted to the mesenteric side of the intestine (Figure 4C, white arrowheads) with little or no staining seen within the antimesenteric mesoderm (Figure 4C, black arrowheads). This suggests an intestine wide effect of the *Fgfr2IIIb* mutation on *Raldh2* expression in addition to the focal down regulation in the atretic precursor.

Discussion

In this paper we performed a characterization of the early morphogenesis of duodenal atresia formation in the *Fgfr2IIIb−/−* mouse model on partially dissected whole mount embryos. We observed changes in the region of the duodenum where the atresia will form as early as E11.5. In this area, a slight narrowing of the duodenum can be seen in *Fgfr2IIIb−/−* embryos. These changes occur in the mouse a full Carnegie stage before an endodermal plug would form in the duodenum of a human. The narrowing becomes more pronounced by E12.5 and by E13.5 in the atretic segment appears to have involuted. Tandler's hypothesis predicts that atresias result from the failure to recanalize the duodenal lumen at CS 18. Thus, the timing of the earliest observed changes to the duodenum in *Fgfr2IIIb−/−* embryos is a full two Carnegie Stages earlier than what would be predicted by Tandler's hypothesis. Given the discrepancy in timing between the two species, these results raise the question as to whether the mechanism of duodenal atresia formation in this animal model involves the formation of an endodermal plug as has been hypothesized for humans. Currently, it is unknown whether mice form an endodermal plug during normal development. A comprehensive analysis of mouse duodenal morphogenesis will be required to determine if this is a possibility.

We also hypothesized that *Fgfr2IIIb−/−* mouse embryos would exhibit disruptions in *Raldh2* expression in the mesoderm during duodenal atresia formation. This was based on several observations. First, that retinoic acid signaling is critical for the development of foregut structures (5, 6). Second, that RA has been shown to interact with Fgf10-Fgfr2IIIb signaling during early lung development (5). Third, that Matthew- Wood syndrome with associated duodenal stenosis arises from mutations in either *FGF10*, *FGFR2* or *STRA6*; suggesting a link between RA signaling and FGF signaling in duodenal development (3, 4). We observed that expression of *Raldh2*, an enzyme that catalyzes the final step in the conversion of vitamin A to the active form retinoic acid, is focally reduced in region of the duodenum where the atresia is forming in comparison to the remainder of the proximal intestine. There are two explanations for this observation. Either *Raldh2* expression is down regulated in the cells that would normally express it or there is a direct loss of the cells that express *Raldh2* during formation of the atresia. To distinguish between these two possibilities would require a Cre that would enable us to perform a lineage tracing to determine the fate of *Raldh2* expressing cells. Currently no such Cre exists. Performing quantitative RT-PCR will not distinguish between these two possibilities either.

This is, however, the first time that the expression of a specific gene within the atretic region of the intestine has be shown to be altered during the formation of this defect. This observation suggests a link between Fgfr2IIIb function and RA pathway, as has been reported in humans (3, 4). We are currently undertaking experiments to assess whether there is a disruption in retinoic acid signaling during atresia formation using a *RARE LacZ* reporter system (9). We are also performing experiments in which vitamin A levels are manipulated through dietary changes in an attempt to alter the severity and penetrance of this defect. Further analysis however will be required to determine whether disruption in retinoic acid signaling is mechanistically important in duodenal atresia formation.

In addition to the focal down regulation seen in the atretic precursor, we observed a general down regulation of *Raldh2* throughout the duodenum and proximal jejunum. What was unique about this altered expression pattern was that it appeared that expression was preserved one on side of the intestine (seemingly the mesenteric side) and lost on the opposite side. Again it is unclear whether this was due to a true down regulation of expression or because of a loss of *Raldh2* expressing cells on one side of the intestine. Either way, the data suggests that there may be an additional phenotype involving RA signaling (aside from atresia) within the intestine of these embryos that has not been previously reported.

The role of disruption in FGFR2IIIb function in the formation of human duodenal atresia is under debate. One recent publication screened a single individual with a duodenal atresia by fluorescent in situ hybridization and failed to identify deletions of *FGF10* and *FGFR2IIIb* (10). The caveat to this study is that it was performed after the atresia was completed and the tissue responsible for formation of the defect was absent. Thus, a somatic mutation in either of these genes within the affected tissue could not be ruled out. In contrast, mutations within the *FGFR2* and *FGF10* genes result in Matthew-Wood syndrome. Humans with this condition have duodenal stenosis, cardiac defects, micrognathia and pulmonary aplasia defects similar to mouse embryos in which both copies of *Fgfr2IIIb* have been mutated (2). Additionally, mutations in the retinol-binding protein receptor gene *STRA6* also result in Matthew-Wood syndrome independent of mutations in either *FGF10* or *FGFR2*. This clinical evidence implicates disruptions in both FGFR2 function and RA signaling in the formation of developmental defects of the duodenum in humans. It also suggests that the *Fgfr2IIIb−/−* mouse embryos are a valid model of the atretic spectrum of defects in human duodenal development.

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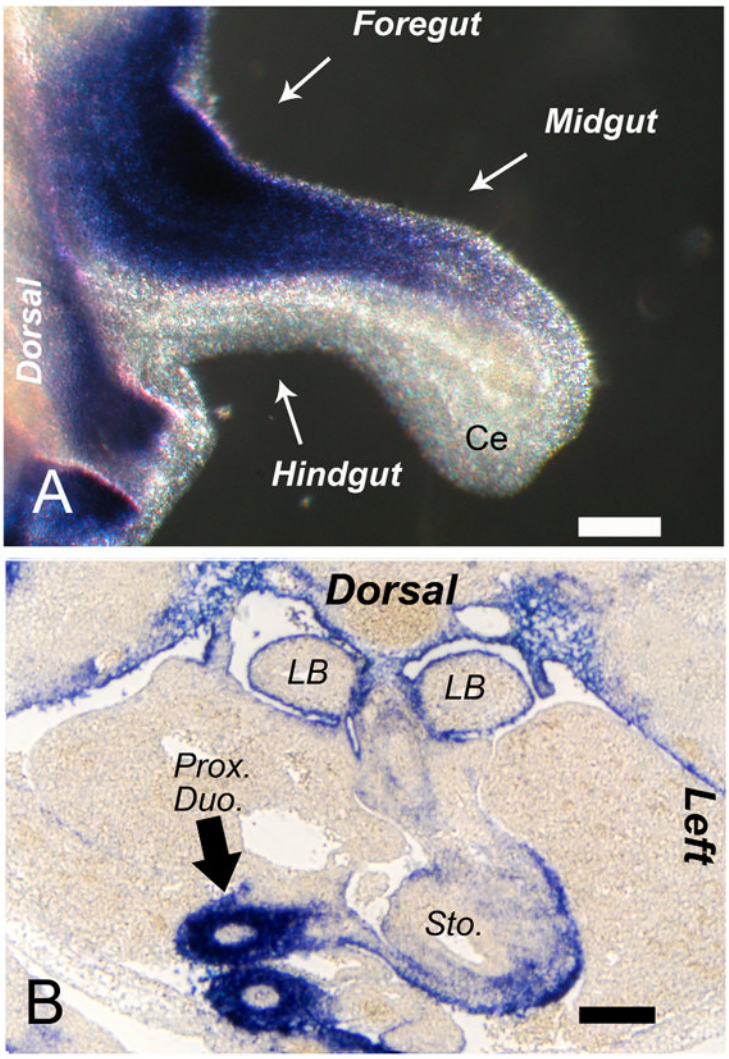

Figure 1.

Morphogenesis in wild-type duodenums (A, C and E) and *Fgfr2IIIb−/−* duodenums developing an atresia (B, D, and F). Black arrow indicates the region where the atresias are forming (the atretic precursor) in the second portion of the duodenum. Narrowing in the atretic precursor is subtle at E11.5 (B) and more evident in the mutant duodenum by E12.5 (D). The defect has gone to completion by E13.5 (F). Stomach and proximal duodenum (Prox. Duo.) are indicated in each image. Measure bars indicate 100 μm.

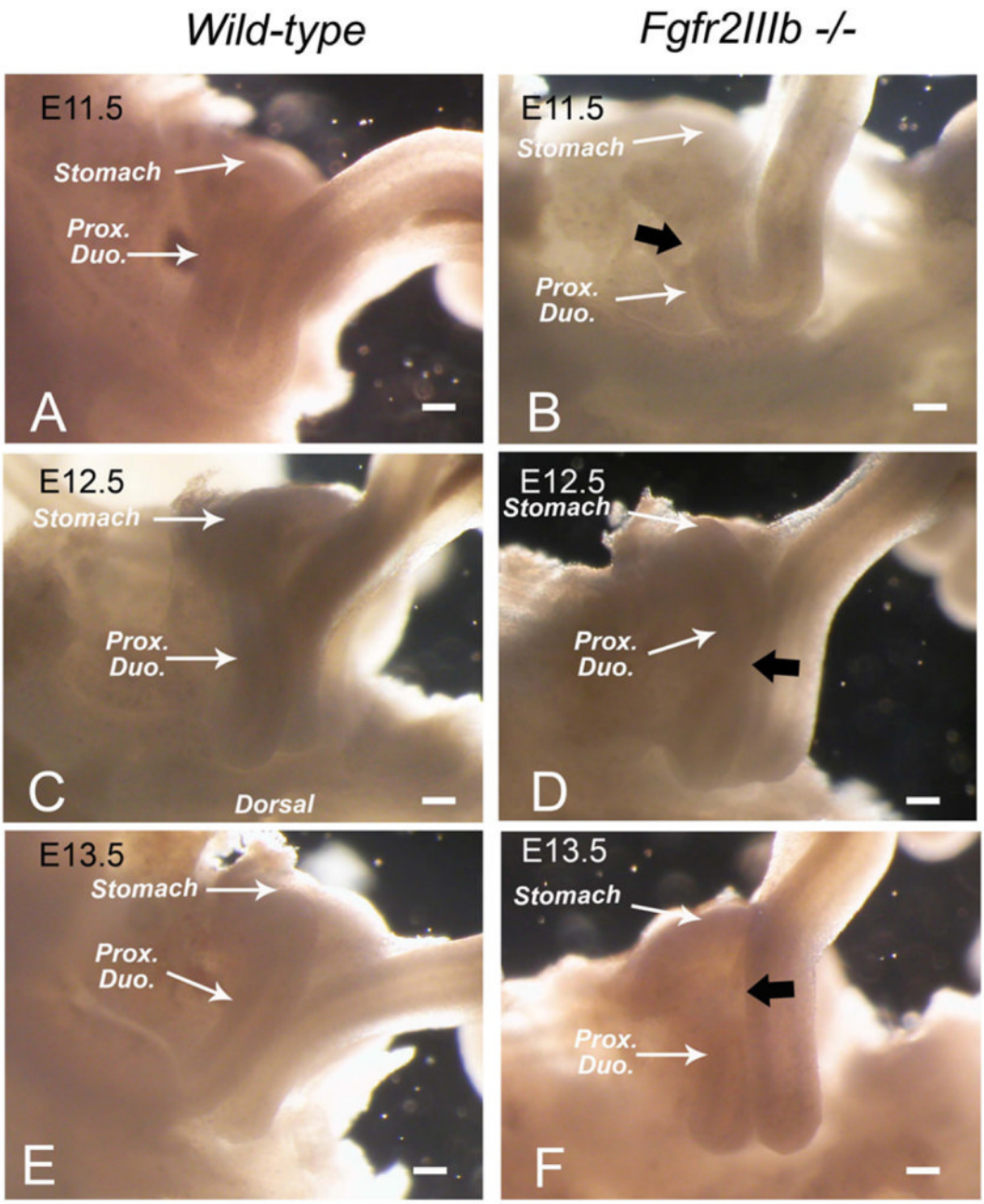

Figure 2.

Expression of *Raldh2* in early duodenal development. A. Whole mount in situ hybridization for *Raldh2* at E11.0. B. Section in situ hybridization of a wild-type mouse embryo at E11.5. The black arrow indicates duodenum. Note the intense staining of the mesoderm and the absence of staining of the endoderm. Lung buds (LB), Stomach (Sto). Measure bars indicate 100 μm.

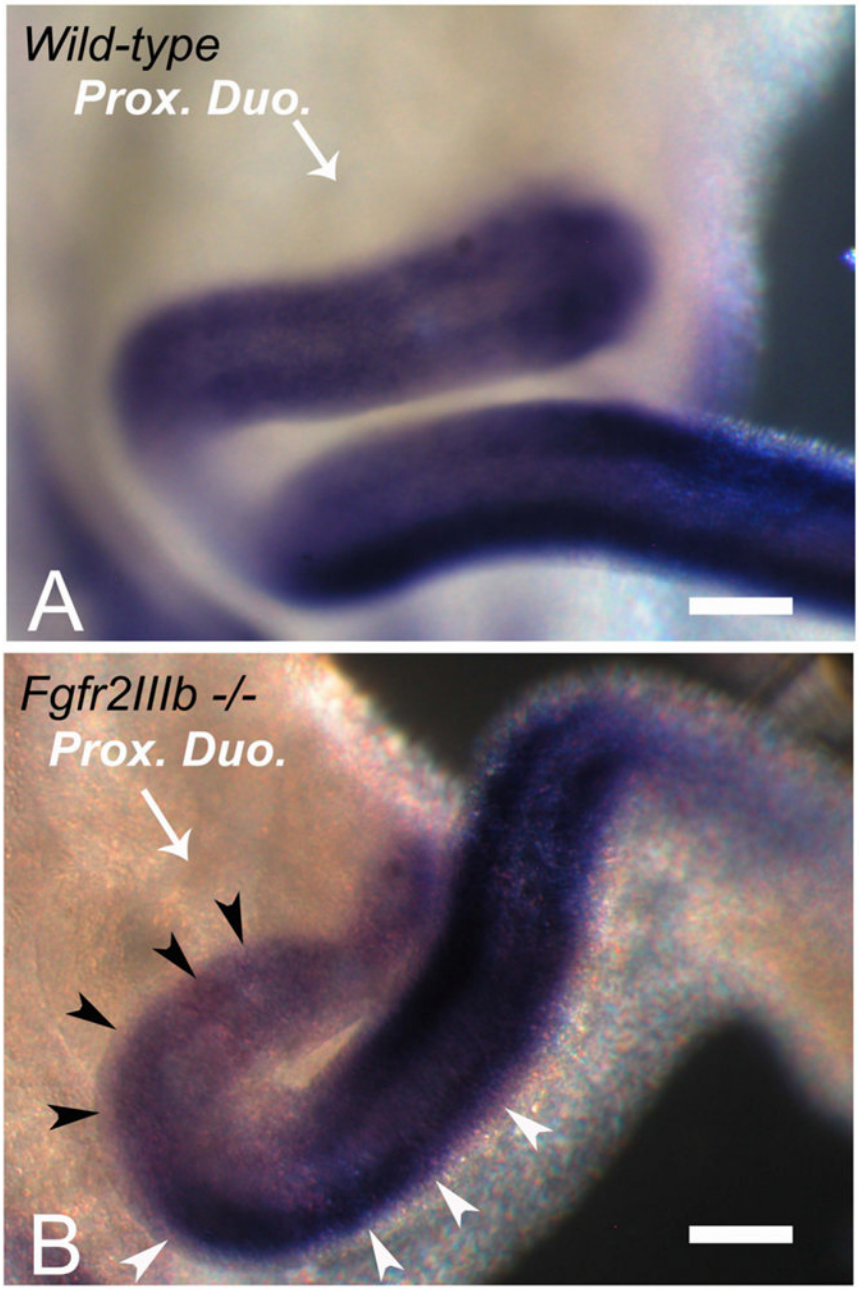

Figure 3.

Expression of *Raldh2* is down regulated in the atretic precursor region of the duodenum of *Fgfr2IIIb−/−* embryos at E11.5. A. Wild-type duodenum demonstrates robust staining in the proximal duodenum. B. Duodenum of an *Fgfr2IIIb−/−* embryo that is developing an atresia demonstrates reduced levels of *Raldh2* in the atretic precursor (Black arrowheads) and robust staining distal to this region (White arrowheads). Measure bars indicate 100 μm.

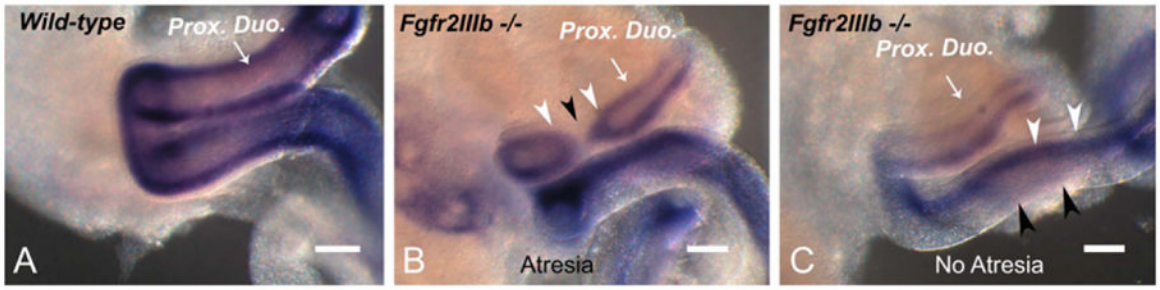

Figure 4.

Expression of *Raldh2* is down regulated during atresia formation in the duodenum of *Fgfr2IIIb−/−* embryos at E12.5 A. Wild-type duodenum demonstrates robust staining in the proximal and distal duodenum. B. Duodenum of an *Fgfr2IIIb−/−* embryo that is developing an atresia demonstrates focal down regulation in *Raldh2* expression in the atretic precursor (Black arrowhead). C. Duodenum of an *Fgfr2IIIb−/−* embryo that is not developing an atresia demonstrates equivalent expression of *Raldh2* in proximal and distal duodenum. However overall expression in the proximal and distal duodenum is restricted to the mesenteric side of the intestine (White arrowheads) compared to the control. Measure bars indicate 100 μm