Title **: The bHLH transcription factor Ascl1a is essential for the specification of the intestinal secretory cells and mediates Notch signaling in the zebrafish intestine**

Running title : Ascl1a specifies the intestinal secretory cell lineage

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Abstract :

Notch signaling has a fundamental role in stem cell maintenance and in cell fate choice in the intestine of different species. Canonically, Notch signaling represses the expression of transcription factors of the *achaete-scute* like (ASCL) or *atonal* related protein (ARP) families. Identifying the ARP/ASCL genes expressed in the gastrointestinal tract is essential to build the regulatory cascade controlling the differentiation of gastrointestinal progenitors into the different intestinal cell types. The expression of the ARP/ASCL factors was analyzed in zebrafish to identify, among all the ARP/ASCL factors found in the zebrafish genome, those expressed in the gastrointestinal tract. *ascl1a* was found to be the earliest factor detected in the intestine. Loss-of-function analyses using the *pia/ascl1a* mutant, revealed that *ascl1a* is crucial for the differentiation of all secretory cells. Furthermore, we identify a battery of transcription factors expressed during secretory cell differentiation and downstream of *ascl1a*. Finally, we show that the repression of secretory cell fate by Notch signaling is mediated by the inhibition of *ascl1a* expression. In conclusion, this work identifies Ascl1a as the master regulator of the secretory cell lineage in the zebrafish intestine, playing the same role as Atoh1 in the mouse intestine. This highlights the diversity in the ARP/ASCL family members acting as cell fate determinants downstream from Notch signaling.

Keywords : Ascl1a; *pia* mutant; Intestine; enteroendocrine; goblet; Notch signalling; ARP; Ascl;

Abbreviations
ASCL, Achaete scute-like; ARP, atonal related proteins; mib, mind bomb; pia, pituitary absent; WISH, whole-mount in situ hybridization; Glp, glucagon-like peptide; pyyb, peptide tyrosine tyrosine b; sst, somatostastin; ghr, ghrelin; hpf, hours post fertilization; dpf, days post fertilization

Introduction

The gastrointestinal tract contains the largest population of hormone-producing cells in the body. Enteroendocrine cells are scattered throughout the digestive epithelium, embedded in a majority of non-endocrine cells, including the absorptive enterocytes and the mucus-secreting goblet cells. They secrete over ten different peptide hormones, regulating important physiological functions such as glycemia, exocrine pancreatic secretion and food intake (reviewed by (Murphy and Bloom, 2006)). Thus, understanding enteroendocrine cell differentiation is essential for identifying future targets for common disease such as diabetes and obesity (reviewed by (Li et al., 2011))

The earliest cell fate decisions for the specification of the different intestinal cell lineages appear to be regulated by the Notch signaling pathway (reviewed by (Schonhoff et al., 2004). In mouse, activation of Notch signaling in the intestine inhibits cell differentiation of all secretory cells, i.e. goblet, Paneth and enteroendocrine cells, resulting in a large increase in the number of progenitor cells (Fre et al., 2005; Stanger et al., 2005). Inversely, inactivation of Notch signaling at adult stage leads to a decrease in the number of progenitors due to excessive differentiation of goblet cells, that populate almost entirely the villi, at the expense of the absorptive enterocytes (Jensen et al., 2000; Pellegrinet et al., 2011; Riccio et al., 2008; van Es et al., 2005). The effect of Notch inactivation on enteroendocrine cells or Paneth cells differentiation is still unclear. Disruption of Notch pathway in Hes1 knockout embryos show also increased numbers of enteroendocrine and Paneth cells (Jensen et al., 2000) while somewhat different results were seen in adult mice treated with γ secretase inhibitors (van Es et al., 2005) or in mice where various components of the Notch signaling pathway were conditionally deleted at adult stages (RBP-jk, Delta1/Delta4, Notch1/Notch2)(Pellegrinet et al., 2011; Riccio et al., 2008; van Es et al., 2005). Disruption of Notch in this context resulted in massive expansion of the goblet cell lineage with little or no effect on the number of enteroendocrine cells and Paneth cells. Such discrepancy could result from a Notch-independent expression of Hes-1 as reported in different systems (Curry et al., 2006; Ingram et al., 2008; Sanalkumar et al., 2010; Wall et al., 2009). Alternatively, different levels of remaining Notch signaling could also explain such discrepancy as the single Delta1 KO has been reported to display an increase of all three types of secretory cells (Stamataki et al., 2011) while in the double KO Delta1/Delta4, only goblet cells are increased (Pellegrinet et al., 2011). Finally, different timings of Notch inactivation could also influence the outcome, therefore it is important to determine the time windows when Notch signaling affects the formation of the different intestinal cell types.

Notch proteins are transmembrane receptors that interact with DSL (Delta Serrate Lag) cell surface ligands from neighboring cells (reviewed by (Schonhoff et al., 2004). Ligand binding activates a series of proteolytic clivages of Notch, releasing from the membrane the Notch intracellular domain that will translocate to the nucleus and activate members of the Hairy Enhancer of Split family. These repressors will then inhibit the expression of cell fate determinants that are usually members of the *Achaete scute-like* (ASCLl) family or of the *atonal* related proteins (ARP) family, this latter family being further subdivided into Atonal, Neurogenin and NeuroD subfamilies. In mouse, several members of these families have been shown to be key regulators of gastrointestinal cell differentiation and, depending of the location in the digestive tract, different sets of cell fate determinants are involved (Jenny et al., 2002; Mellitzer et al., 2010; Shroyer et al., 2007; Yang et al., 2001). For example, ASCL1 (Mash1) is absolutely required for the differentiation of all endocrine cells in the stomach (Kokubu et al., 2008) but is not expressed in the intestine (Jensen et al., 2000; Kokubu et al., 2008) while ATOH1 (Math1) is absolutely required for enteroendocrine and goblet cell differentiation in the intestine (Shroyer et al., 2007; Yang et al., 2001) but is not expressed in the stomach (Yang et al., 2001). Ngn3 is the cell fate determinant of the endocrine lineage in the intestine (Jenny et al., 2002; Mellitzer et al., 2010) but is important for only a subsets of enteroendocrine cells in the stomach. Many unanswered questions still remain regarding notably how the different enteroendocrine subtypes are specified and what are the factors or signaling events that control the choice between goblet and enteroendocrine cell fate. Identifying all transcription factors involved in these processes and understanding the spatial and temporal expression of these factors during intestinal differentiation is required in order to build the regulatory cascade controlling the differentiation of gastrointestinal progenitors into secretory cells.

The analysis of Notch function in the same organ of different species is also valuable as it allows exploiting the specific advantages of each model system to highlight both conserved and divergent functions across species (Fre et al., 2011). One of the advantages of the zebrafish model is the possibility to visualize the expression profile of a particular factor at once in the whole animal by whole-mount in situ hybridization (WISH) and to analyze easily in the whole digestive system the phenotypic defects caused by its loss-of-function. The functional organization of the zebrafish digestive system is similar to mammals; however, its morphology is simpler. The zebrafish gastrointestinal tract does not have a discrete stomach but the anterior part of the gut tube is enlarged to form a food-storage compartment known as the intestinal bulb. This bulb and the adjacent mid-intestine are involved in lipid absorption. Most of the posterior intestine is an absorption zone for proteins while the caudal end is specialized in ion transportation and water absorption, like the colon

of mammals. There are no 'crypts of Lieberkuhn' in the zebrafish intestinal epithelium but intervillus pockets which are, as the crypts, the site of cell proliferation required for the intestinal turnover but which do not contain Paneth cells. The villi contain three types of differentiated cells, namely, enterocytes, goblet and enteroendocrine cells (Crosnier et al., 2005; Wallace et al., 2005).

Like in mammals, Notch signaling plays an essential role in the control of intestinal homeostasis in zebrafish. Indeed, the *brom bones* mutants, that exhibit increased Notch signaling, display hyperproliferation of the adult intestinal epithelium and compromised secretory goblet cell differentiation; however the consequence on enteroendocrine cell differentiation was not investigated (Yang et al., 2009). Inversely, in the *mind bomb* (*mib)* mutants where the Notch signaling pathway is inactivated, almost all the cells in the 3-day gut epithelium adopt a secretory fate as revealed by the monoclonal antibody 2F11, that recognizes both enteroendocrine and goblet cells (Crosnier et al., 2005). A role of Notch on enteroendocrine cell differentiation is suggested by an increase of Glucagon-like peptide (Glp) expressing cells in the *mib* mutant (Zecchin et al., 2007). The question whether both goblet and enteroendocrine cell fates are favored in the *mib* mutant remains unanswered.

In this study, we analyzed the effect of Notch inactivation on the different secretory cell types of the zebrafish intestine and determined the critical time window of Notch action. Moreover, we identified *ascl1a* as the first ARP/ASCL factor expressed in the gastrointestinal tract and found that it acts directly downstream of Notch signaling to promote secretory cell fate.

Materials and Methods

Zebrafish maintenance and mutant lines

Zebrafish (*Danio rerio*) were raised and cared for according to standard protocols (Westerfield, 1995). Wild-type embryos from the AB strain were used and staged according to Kimmel (Kimmel et al., 1995). The *pia* mutant (*pia*^{t25215} mutant allele of *ascl1a*) was provided by M. Hammerschmidt and genotyped as described (Pogoda et al., 2006). Homozygous *mind bomb* mutants were obtained by mating heterozygous fish for the *mib*^{ta52b} allele (Haddon et al., 1998)

Riboprobes, Whole-mount *in situ* hybridization (WISH)

Antisense riboprobes were made by transcribing linearized cDNA clones with SP6, T7, or T3 polymerase using digoxigenin or DNP labeling mix (Roche) according manufacturer's

instructions. They were subsequently purified on NucAway spin columns (Ambion) and ethanol-precipitated. The zebrafish *fabp2* (Mayer and Fishman, 2003) *agr2* (Shih et al., 2007), a*scl1a* (Allende and Weinberg, 1994), *ascl1b* (Allende and Weinberg, 1994), *sox4b* (Mavropoulos et al., 2005), *isl1* (Korzh et al., 1993), *neurod1* (Korzh et al., 1998), *pax6b* (Krauss et al., 1991), *rfx6* (Soyer et al., 2010), *ngn3* (Wang et al., 2001), nkx2.2 (Barth and Wilson, 1995), *somatostatin2* (Devos et al., 2002), *ghrelin* (NCBI: AL918922), *pyyb* (GenBank: AL909489) and *gcga* (Argenton et al., 1999) probes have been described elsewhere. Single whole-mount and double fluorescent *in situ* hybridizations were carried out as described (Mavropoulos et al., 2005).

Immunochemistry

Whole-mount immunohistochemistry were performed as previously described (Dong et al., 2007). We used the monoclonal mouse 2F11 primary antibody (Crosnier et al., 2005) (1:1000, abcam ab71286), the mouse monoclonal mouse 4E8 primary antibody (1:150, abcam ab73643) (Crosnier et al., 2005), and fluorescently conjugated Alexa-488 antibody for detection (Molecular Probes). Goblet cell mucin was detected with rhodamine conjugated wheat germ agglutinin (1:100 dilution) (Vector Laboratories). The nuclei were stained by TO-PRO-3 iodide (642/661 nm, Invitrogen) or by Draq7™ (Biostatus limited) and embryos were mounted in ProLong Gold Antifade reagent (Invitrogen).

Imaging

Microscope pictures were performed with an Olympus DP70 photocamera fixed on a BX60 Olympus microscope. Confocal imaging was performed using a Leica TCS SP2 inverted confocal laser microscope (Leica Microsystems, Germany). Digitized images were acquired using a 63X (NA 1.2) Plan-Apo water-immersion objective at 1024X1024 pixel resolution. For multicolour imaging, FITC was visualized by using an excitation wavelength of 488 nm and the emission light was dispersed and recorded at 500-535 nm. Cy3 was detected by using an excitation wavelength of 543 nm and the fluorescence emission was dispersed and recorded at 555-620 nm. TO-PRO-3 iodide and Draq7™ were detected by using an excitation wavelength of 633 nm and the fluorescence emission was dispersed and recorded at 650-750 nm. The acquisition was set up to avoid any cross-talk of the two fluorescence emissions. Series of optical sections were carried out to analyze the spatial distribution of fluorescence, and for each embryo, they were recorded with a *Z*-step ranging between 1 and 2 µm. Image processing, including background subtraction, was performed with Leica software (version 2.5). Captured images were exported as TIFF and further processed using Adobe Photoshop and Illustrator CS2 for figure mounting.

Morpholino design and injection

The *sox4b* morpholino was designed by Gene Tools and is complementary to the 5'UTR (5'-GACTCAGTCTGATTGCACACAGTCC-3'). A standard control MO, having the sequence: 5'-CCTCTTACCTCAGTTACAATTTATA 3' has been also designed by Gene Tools in a way that it should have no target and no significant biological activity. They were dissolved at a concentration of 3 µg/µl in 1x Danieau buffer containing 0,5 % of rhodamine dextran (to follow the microinjection process) and microinjected at the 1-2 cells stage at a dose of 6 ng. Injected embryos were then grown in the presence of 0.003% 1-phenyl-2-thiourea until the desired stage, fixed overnight in 4% PFA and stored in 100% methanol before analysis.

Drug inhibition of Notch-signaling in larvae

N-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT, D 5942, Sigma), a presenilin γ-secretase inhibitor was used to block Notch-signalling. A 100× stock solution of 10 mM DAPT in DMSO was made and stored at −20 °C until use. Embryos were dechorionated and incubated in 100 µM of DAPT in E3 (Geling et al., 2002) . Embryos incubated in 1% DMSO in E3 were used as control.

Results

ascl1a is expressed in the precursor cells of the gastrointestinal tract

As cell-fate commitment controlled by Notch signaling is canonically carried out via ARP/ASCL factors, we searched for ARP/ASCL factors that would act as cell fate determinants in the gastrointestinal tract. Among the 14 ARP/ASCL factors found in the zebrafish genome (Wang et al., 2009), we found that *ascl1a* was the first to be expressed in the gastrointestinal tract. In addition to its previously reported expression in the nervous system and in the adenohypophysis (Allende and Weinberg, 1994; Pogoda et al., 2006), we found a strong expression of *ascl1a* in the intestine (Figures 1 et S1) while its paralog *ascl1b* was not detectable in this tissue (data not shown). The expression of *ascl1a* in the primitive gut begins at 36 hpf in few scattered cells and become easily detectable at 40 hpf (Figure 1B). *ascl1a* shows a punctuate distribution in the intestinal epithelium (see Supplemental figure S1), which consists at that stage of a thin layer of cuboidal cells (Ng et al., 2005). As previously reported (Lucas et al., 2006), the enteric neuron progenitors found in the mesenchyme surrounding the intestinal epithelium also express *ascl1a* (arrows in Figure 1B). At 58 hpf, the anterior region of the intestinal epithelium has expanded to form the intestinal

bulb and cells expressing *ascl1a* remain scattered throughout the primitive gut (Figure 1C). The intestinal expression of *ascl1a* begins to decrease at 72 hpf (Figure 1D), time where the first hormones begin to be expressed by the enteroendocrine cells (i.e *pyyb, gcga*) (Ng et al., 2005). Finally, *ascl1a* expression disappears shortly after 84 hpf.

Double fluorescent *in situ* hybridization using *ascl1a* and *gcga* (processed into Glp in intestinal cells) (Figure 1E) or *peptide tyrosine tyrosine b* (pyyb) probes (data not shown) did not reveal any co-localisation between *ascl1a* and these two hormones. Moreover, we did not observe any co-expression of *ascl1a* with *agr2,* a marker of mature goblet cells, coding for a protein disulfide isomerase essential for Mucin2 maturation (Shih et al., 2007) (Figure 1F). The absence of *ascl1a* expression in mature secretory cells and its precocious expression in the intestinal epithelium suggest that *ascl1a* could label precursor cells in the gastrointestinal tract.

Absence of the secretory lineage in the intestine of *ascl1a/pia* mutants

To determine the role of Ascl1a in intestine development, we analyzed the phenotype of the *pituitary absent (pia)* mutant, carrying a null mutation within the *ascl1a* gene (Pogoda et al., 2006). This point mutation is a nucleotide substitution changing the tyrosine codon at position 70 to a premature stop codon resulting in a truncated Ascl1a protein that lacks all the C-terminal amino acid residues including the entire bHLH domain.

In wild-type embryos, expression of *pyyb* was mainly detected in enteroendocrine cells of the intestinal bulb while *gcga* expression is essentially found in enteroendocrine cells of the mid and posterior gut (Figure 2A, C and see upper scheme). In the *pia* mutant, neither *pyyb* nor *gcga* expression was detectable in the gastrointestinal tract (Figure 2 B, D). We next investigated goblet cells distribution in the gut epithelium by WISH using an *agr2* probe or by the binding of a fluorescently labeled wheat germ agglutinin (WGA) that interacts with the N-acetylglucosamine of the mucus (Wallace et al., 2005). Goblet cells were easily detected in the middle and posterior segments of the intestine of wild-type larvae at 84 hpf and 120 hpf, (Figure 2 E, G). In contrast, no goblet cells were found in the *pia* larvae (Figure 2 F, H). The absence of goblet cells and of *pyyb*- or *gcga*-enteroendocrine cells indicate that the secretory lineage is severely affected in the *pia* mutants. To determine whether the whole secretory lineage is perturbed, we performed an immunolabelling using the antibody 2F11 that recognizes both goblet and enteroendocrine cells (Crosnier et al., 2005) (Figure 2I). No 2F11 positive cells were present in the gastrointestinal tract of the *pia* mutant (Figure 2J). In contrast, the absorptive lineage seems unaffected as enterocytes, labeled by *fabp2 (fatty acid binding protein 2, intestinal)* probes, were detected in the *pia* mutants like in the wild-

type siblings (Figure 2 K, L). This conclusion was further supported by immunohistochemistry using the 4^{E}8 antibody, described to stain the brush border of the absorptive cells (Crosnier et al., 2005). As shown on supplemental figure S2 C-D, the brush border of the enterocytes seems to be correctly formed in the *pia* mutant at 5 dpf. Furthermore, the general morphology of the digestive tract does not appear disturbed as revealed by the TO-PRO-3 iodide labeling of the cell nuclei (Figure S2 A-B).

All these data indicate that, during zebrafish embryogenesis, Ascl1a is essential for the formation of all intestinal secretory cells but not for the generation of the absorptive cells.

Ascl1a is a master regulator of the secretory cell differentiation cascade

To date, there are only a few transcription factors known to be expressed in the secretory lineage in the zebrafish intestine. To our knowledge, only *pax6b, ngn3, nkx2.2* and *neurod1* were reported as expressed in scattered cells of the gut (Delporte et al., 2008; Ng et al., 2005; Zecchin et al., 2007). These factors are known to be important for enteroendocrine cell differentiation in mice (Desai et al., 2008; Hill et al., 1999; Jenny et al., 2002; Larsson et al., 1998; Lee et al., 2002; Mutoh et al., 1997; Mutoh et al., 1998; Naya et al., 1997; Schwitzgebel et al., 2000). These four factors are also involved in pancreatic endocrine cell differentiation, suggesting that a similar regulatory cascade controls the differentiation of endocrine cells in both the pancreas and the intestine. This observation led us to analyze the expression of other pancreatic transcription factors in the gastrointestinal tract. Three additional transcription factors, namely *sox4b, isl1* and *rfx6* were detected in scattered cells of the intestine (Figure 3 E, G, I). Expression of these seven pancreatic transcription factors was analyzed at different time points during gastrointestinal differentiation in order to define the onset of their expression and their distribution in the gut (see recapitulative scheme in Figure 3A). We found that the transcription factor *sox4b*, which is known to be a specific marker of endocrine precursors in the pancreas, begins to be expressed in few cells of the gut at 36 hpf and is easily detectable at 40 hpf like *ascl1a*. Furthermore, *sox4b* was found to co-localize with *ascl1a* at all examined stages (40 hpf, 48 hpf, 58 hpf) (Figure 3B-D and data not shown). Expression of *neurod1, nkx2.2, rfx6, pax6b* and *isl1* was detected between 50 and 65 hpf (Figure 3A). *ngn3* expression was not detected before 72 hpf and only in few cells of the gastrointestinal tract (supplemental Figure S3, G). Its onset of expression occurs approximately at the same time as the hormones *pyyb*, *ghrelin*, *gcga* and *somatostatin*. Finally, *agr2*, a marker of mature goblet cells (Shih et al., 2007) is easily detected from 84 hpf onwards.

None of these seven transcription factors were detected in the gastrointestinal tract of the *pia* mutants (Figure 3 F, H, J and S3 A-H). In contrast, the expression of all these factors

was not perturbed in the pancreas indicating that Ascl1a controls endocrine cell differentiation in the intestine but not in the pancreas. The absence of pancreatic effect is not surprising as we found that *ascl1a* is barely detectable in a small number of pancreatic cells (data not shown). To further analyze the genetic hierarchy between Ascl1a and Sox4b, we analyzed *ascl1a* expression after Sox4b inactivation. The injection of the antisense morpholino Mo1sox4b, targeting the 5'UTR of *sox4b* mRNA and known to efficiently block *sox4b* translation, does not affect *ascl1a* expression at 55 hpf (Fig. 3 K, L) while inhibiting the pancreatic expression of glucagon as previously reported (Mavropoulos et al., 2005).

All these data indicate that Ascl1a acts at the beginning of the intestinal secretory cell differentiation cascade and is a master regulator playing a pivotal role in the specification of the secretory cell lineage.

Ascl1a acts downstream of Notch signaling to promote secretory cell fate

In order to establish the genetic relationship between Notch signaling and Ascl1a, we analyzed the expression of *ascl1a* in the *mib* mutant where the Notch signaling pathway is inactivated (Itoh et al., 2003). *mib* embryos display a strong increase in the number of *ascl1a*-expressing cells with almost all intestinal cells expressing that gene. In contrast, the wild-type embryos show a "salt and pepper" distribution, as classically found for genes repressed by the Notch lateral inhibition process (Figure 5A).

Previous analyses of *mib* mutant larvae has revealed that almost all the cells in the 3-day gut epithelium adopt a secretory fate (Crosnier et al., 2005); however, it was not determined whether both goblet and enteroendocrine fate is adopted in these mutants. Thus, we performed an immunohistochemistry using the monoclonal antibody 2F11, that recognizes both enteroendocrine and goblet cells, combined with the binding of fluorescent WGA labeling the goblet cells. As shown on figure 4F, we found *in mib* larvae a nearly complete loss of *fabp2*-labeled enterocytes. These data confirm previous reports showing a dramatic reduction of absorptive cells labeled with the brush border antibody $4^{E}8$ (Crosnier et al., 2005) or with the oligopeptide transporter PepT1 probe (Zecchin et al., 2007). The gut of *mib* mutants, which is reduced in size, is nearly completely covered by secretory cells (Figure 4B), either enteroendocrine cells (2F11+/WGA-), found preferentially in the bulb and the midgut, or goblet cells (2F11+/WGA+). The presence of goblet cells in the gut of *mib* mutant was confirmed by WISH using the agr2 probe (Figure 4D).

To define the time window when Notch signaling is required for repressing the secretory fate, we treated wild-type embryos with DAPT, a γ-secretase inhibitor of Notch signaling pathway (Geling et al., 2002). The number of enteroendocrine cells was determined at 75 hpf

by WHISH using a cocktail of probes for four hormones (*pyyb, gcga, ghrelin* and *somatostatin*). Treatments starting at 25 or 30 hpf (25 to 75 hpf or 30 to 75 hpf) led to a significant increase in the number of enteroendocrine cells (Figure 5B, C) while treatment initiated at 40 hpf or 45 hpf had no effect on the number of enteroendocrine cells (Figure 5B). When the treatment began at 35 hpf, a moderate increase in the number of endocrine cells was observed (Fig. 5B). All these data indicate that Notch signaling represses enteroendocrine cell fate in a limited time window that occurs between 25 and 40 hpf during embryonic development.

To determine whether the repression of the enteroendocrine cell fate mediated by Notch signaling occurs through the regulation of *ascl1a* expression, we first compared *ascl1a* expression in embryos treated with DAPT from 25 hpf to 55 hpf versus embryos treated from 40 to 55 hpf (Figure 5 D-E). Consistent with the moment when Notch is required, the number of *ascl1a*-expressing cells was increased only with the first treatment suggesting that the activity of Notch signaling could be mediated through the regulation of *ascl1a* expression. To further support this assertion, we investigated the consequence of *ascl1a* loss-of-function combined with Notch inhibition by treating the *pia* mutants with DAPT from 25 hpf to 96 hpf. Inhibition of Notch signaling in wild-type embryos results in an excessive differentiation of enteroendocrine cells and goblet cells (Figure 6 A-A', C-C'). In contrast, no secretory cells were observed in the *pia* mutants with or without Notch signaling inhibition (Figure 6 B-B', D-D'). This demonstrates that the induction of the secretory cell fate upon disruption of Notch signaling requires Ascl1a.

Taken together, all these data indicate that Notch signaling represses secretory cell fate by inhibiting *ascl1a* expression.

Discussion

Our findings provide new insights into the role of Notch in controlling differentiation of intestinal epithelial lineages during zebrafish embryogenesis. We found that Notch signaling represses both goblet and enteroendocrine cell differentiation via the repression of *ascl1a* expression. *ascl1a*, expressed in the intestinal precursors at early stages, is indispensable for the specification of the secretory cell lineage. It acts upstream of a battery of transcription factors, thereby placing it at the top of the secretory cell differentiation cascade.

ascl1a is the first _ARP/Ascl_ gene expressed in the gastrointestinal tract.

Among the 14 ARP/ASCl genes found in the zebrafish genome (Wang et al., 2009), *ascl1a* is the first to be expressed in the gastrointestinal tract. *ascl1a* is expressed in scattered cells of the epithelium of the gastrointestinal tract, long before the apparition of the first mature intestinal cells, and never co-localizes with markers of mature secretory cells. The disseminated distribution of *ascl1a* is a sign of lateral inhibition, which allows the generation of secretory cells scattered within the intestinal epithelium. This 'salt and pepper' distribution of *ascl1a* positive cells and the loss of all secretory cells in *ascl1a* mutants suggests that *ascl1a* is expressed in all precursors of the secretory cell lineage but not in the enterocytes precursors. Furthermore, *sox4b*, which is a marker of endocrine cell precursors in the pancreas, is co-expressed with *ascl1a* in the intestine at all tested stages. This suggests that both *sox4b* and *ascl1a* are markers of secretory lineage precursors. However, cell lineage experiments are required to definitively prove this hypothesis.

Many pancreatic transcription factors are also expressed in the intestine.

There is a striking similarity in the transcription factors expressed in the endocrine cells of the pancreas and of the intestine. Indeed, several transcription factors expressed in the enteroendocrine cells in mouse and zebrafish, such as Pax6 (Delporte et al., 2008; Hill et al., 1999; Larsson et al., 1998), Ngn3 (Jenny et al., 2002; Lee et al., 2002; Schwitzgebel et al., 2000; Zecchin et al., 2007), Neurod1 (Mutoh et al., 1997; Mutoh et al., 1998; Zecchin et al., 2007), Nkx2.2 (Desai et al., 2008; Ng et al., 2005), Isl1 (Larsson et al., 1995) (this study), are also essential for pancreatic endocrine differentiation (Kinkel and Prince, 2009; Rojas et al., 2010). Moreover, we found that two other pancreatic factors, *sox4b* and *rfx6,* are also expressed in the zebrafish intestine. The detailed analysis of the expression profile of these seven factors allowed us to establish the order of their apparition, giving us a hint about the regulatory cascade driving enteroendocrine cell differentiation in zebrafish. The first transcription factors that appear in the gastrointestinal tract are *ascl1a* and *sox4b* which are detectable from 36 hpf onwards. Then, the factors *neurod1, nkx2.2, rfx6, pax6b* and *isl1* appear between 50 and 65 hpf and finally *ngn3* whose expression is detectable only from 72 hpf, when the first enteroendocrine cells are already differentiated (see diagram Figure 3A). The relatively late onset of *ngn3* expression in the zebrafish gastrointestinal epithelium is not in favor of a role of this factor as cell fate determinant of the enteroendocrine cell lineage. In mice intestine, *Ngn3* is expressed long before the first enteroendocrine cells are differentiated and is required for the differentiation of all these cells (Jenny et al., 2002). In contrast, in the stomach, *Ngn3* expression is only involved in the differentiation of a subset of enteroendocrine

cells (Jenny et al., 2002; Lee et al., 2002). As *ngn3* expression appears at late stages in zebrafish, it is possible that it affects only the subpopulation of enteroendocrine cells appearing at later stages, such as GIP, GRP, NPY or PYY expressing cells (Musson et al., 2009)(unpublished data).

Notch signaling represses the secretory cell fate by inhibiting *ascl1a* expression.

In zebrafish as in mouse, secretory cell differentiation is controlled by Notch signaling. In mouse, Notch activation inhibits cell differentiation of all intestinal secretory cells (Fre et al., 2005; Stanger et al., 2005) while Notch inhibition leads to an excessive differentiation of goblet cells (Jensen et al., 2000; Pellegrinet et al.; Riccio et al., 2008; van Es et al., 2005). The actual role of Notch pathway in the differentiation of the murine enteroendocrine and Paneth cells is still unclear. In zebrafish, Notch signaling activation using the *brom bones* mutant shows a defect in goblet cell specification in adult but the effect on enteroendocrine cells was not investigated (Yang et al., 2009). Notch signaling inactivation using the *mib* mutants revealed that almost all the cells in the gut epithelium adopt a secretory fate, either goblet or enteroendocrine fate (this study, (Crosnier et al., 2005; Zecchin et al., 2007). As a consequence of the loss of absorptive cells, *ascl1a* staining is found in nearly all the cells of the gut of the *mib* mutant. DAPT treated embryos show also an increase of *ascl1a* expressing cells but the cells remain scattered in the gut probably due to the presence of enterocytes in the DAPT- treated embryos. Such differences between *mib* mutant and DAPT treated embryos can be explained by either an incomplete blocking of Notch activity by the DAPT treatment or by the different timing of Notch inactivation. Indeed, DAPT treatment was performed only from 25 hpf onwards while the early inactivation of Notch signaling in the *mib* mutant can affect additional processes like endoderm formation (Kikuchi et al., 2004).

DAPT treatment during different time intervals indicated that Notch signaling pathway represses enteroendocrine cell differentiation during a short time window (25hpf-40 hpf). This time interval precedes the onset of intestinal *ascl1a* expression. As DAPT treatments must be performed during the same time interval to trigger *ascl1a* upregulation, this strongly suggests that Ascl1a is the mediator of Notch signaling in the zebrafish intestine. This was confirmed by the fact that Ascl1a is absolutely required for the induction of secretory cell fate upon Notch inactivation. All together, these data indicate that, during zebrafish embryogenesis, Notch signaling represses the secretory cell fate by inhibiting *ascl1a* expression. The question if this is also the case during the continuous cell renewal of the intestinal cells at late developmental stages and during adulthood still remains to be addressed.

Diversity in the ARP/Ascl factors involved in the specification of the endocrine cells of the gastrointestinal tract.

In the mouse intestine, the cell fate determinant of the secretory lineage is Atoh1 (Shroyer et al., 2007; Yang et al., 2001). In zebrafish, the *Atoh1* orthologs, *atoh1a* and *atoh1b* are respectively not expressed or at very low level in the gastrointestinal tract (data not shown) and, as shown in this study, the role of secretory cell fate determinant is held by Ascl1a. This is quite surprising at first glimpse since these two factors belong to distinct families, namely the ATO and the ASCL group. However, such cross-species diversity in the choice of ARP/ASCL factors exists also in other tissues. For example, the group of ATO factors efficiently induces neurogenesis in Drosophila, but very weakly in Xenopus; inversely, NGN group proteins are potent neural inducers in Xenopus but extremely weak inducers in flies (Quan et al., 2004). This diversity is also observed across organs in the same species. For example, Ngn3 is the cell fate determinant of the endocrine lineage in the murine pancreas and intestine (Jenny et al., 2002; Mellitzer et al., 2010) but not in the stomach where ASCL1 plays that role (Kokubu et al., 2008). This highlights the diversity in the choice of the ARP/ASCL determinants within a highly conserved mechanism of cell fate selection controlled by Notch signaling and this diversity depends not only on the organ considered but also on the species.

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Figure legends:

Figure 1: *ascl1a* is expressed in the precursor cells of the gastrointestinal tract. Ventral views with anterior on the left of embryos analyzed by visible WISH (A-D) or double fluorescent WISH (E-F). **(A)** General view of *ascl1a* expression domain at 55hpf. **(B)** At 40hpf, *ascl1a* is expressed in cuboïdal cells scattered in the primitive intestine (dotted lines) and in enteric neurones that lined these cells (arrows). **(C)** At 58 hpf, this scattered expression is still intense. **(D)** At 72hpf, the number of *ascl1a* expressing cells begins to decrease along the gastrointestinal tract. (**E-F)** Confocal section showing that *ascl1a* is not expressed in the mature enteroendocrine cells labelled by the *gcga* probe (E) neither in the mature goblet cells labelled by *agr2* (F). Scale bars : 50 µM

Figure 2: Absence of the secretory lineage in the *ascl1a/pia* mutant. Upper part: Scheme depicting the organisation of the zebrafish gastrointestinal tract. Lower part: Confocal projections of ventral views of embryos with the anterior to the left analyzed by fluorescent WISH (A-F,K-L), labelling with a rhodamine dextran-conjugated wheat germ agglutinin (WGA) (G,H) or by 2F11 immunohistochemistry (I-J). **(A-F)** The *pia* mutant lack the enteroendocrine cells expressing *pyyb* (B) or *gcga* (D) as well as the goblet cells expressing *arg2* (F). **(G-H)** Goblet cell mucin, revealed by rhodamine-dextran labelled WGA, is absent in the *pia* mutant. **(I-J)** Immunohistochemistry with 2F11 antibody confirms that the whole secretory lineage is missing in the gastrointestinal tract (dotted lines) of the *pia* mutant. **(K-L)** The absorptive cells, labelled with the *fabp2* probe, are still present in the *pia* mutant. Scale bars : 50 µM

Figure 3: Ascl1a is a master regulator of the secretory cell differentiation cascade. (A) Diagram illustrating the expression window of the transcription factors and hormones in the gastrointestinal tract. The interval of time where the gene has been shown to be expressed is represented by a solid line whereas the gene expression was not analysed during the periods outlined by dotted lines.

The references for these data are as follows : a (this paper), b (Ng et al., 2005), c (Zecchin et al., 2007), d (Delporte et al., 2008), e (Wallace et al., 2005). **(B-L)** Ventral views with anterior to the left of embryos analyzed by fluorescent WISH (B-J) or visible WISH (K-L) **(B-D)** Confocal sections showing that *ascl1a* expression co-localize perfectly with *sox4b* expression in scattered cells of the primitive intestine (dotted line) at 48hpf. **(E-J)** Confocal projections showing that all the gastrointestinal cells expressing *sox4b*, *isl1* or *rfx6* are absent in the *pia* mutant whereas the pancreatic expression of these factors is not affected **(K-L)** *ascl1a* expression is not affected upon Sox4b knock-down. p, pancreas; Arrow, enteric neurone. Scale bars : 50 µM

Figure 4: All the cells of the gut epithelium adopt either a goblet or enteroendocrine fate in the *mib* mutant.

Ventral views with anterior to the left of *wt* or *mib* embryos labelled by immunohistochemistry with 2F11 together with the binding of fluorescent WGA (A-B) or by WISH using the *agr2* probe (C-D) or the *fabp2* probe (E-F). A-B: The gut of 5-day *mib* mutants is nearly completely covered by secretory cells, either enteroendocrine cells (2F11+/WGA-), found preferentially in the intestinal bulb and the midgut, or goblet cells (2F11+/WGA+). C-D: The presence of goblet cells in the gut of *mib* mutant at 4 dpf was confirmed by WISH using the agr2 probe. The double-headed arrows indicate the length of

the gut. E-F: 84 hpf *mib* larvae display a nearly complete loss of *fabp2*-labeled enterocytes. Scale bars: 50 µM

Figure 5: Notch signalling acts between 25 hfp and 40 hpf to repress enteroendocrine cell differentiation and *ascl1a* expression. Ventral views with anterior to the left of embryos analyzed by visible (A,D-G) or fluorescent (C) WISH. **(A)** *ascl1a* expression is strongly increased in the primitive gut (dotted lines) of 45 hpf *mib* embryos. **(B)** Quantification of the total number of enteroendocrine cells present in the whole intestine and labelled with a mixture of *pyyb, gcga*, *somatostatin (Sst*) and *ghrelin (Ghr)* probes. These positive cells have been directly counted under the microscope. Fold increase represents the mean (+ S.EM.) of the enteroendocrine cells number in DAPT treated embryos versus DMSO treated embryos. Asterisks indicate that the difference between the cell number in control and embryos treated with DAPT is statistically significant by Student's *t*-test. (***) *P*<0.0001; (**) P<0,001; (*) P<0,05. **(C)** Confocal projections of 75 hpf embryos labelled with a mixture of *pyyb, gcga*, *somatostatin (Sst*) and *ghrelin (Ghr)* probes and treated with DAPT or DMSO from 30hpf-75hpf. P, pancreas **(D-G)** *ascl1a* expression at 55 hpf is increased in the primitive gut of embryos treated with DAPT from 25 to 55 hpf (E) but not in embryos treated from 40 to 55hpf (G).

Figure 6: Ascl1a is required for the induction of secretory cell fate upon inhibition of the Notch pathway. Ventral views with anterior to the left of embryos analyzed by double fluorescent WISH using a *agr2* probe (green) and a mix of *pyyb*, *gcga*, *somatostatin* (Sst) and *ghrelin* (Ghr) probes (red). All pictures are confocal projections. (A,C) WT embryos treated with DAPT from 25 to 96hpf show an increased number of enteroendocrine and goblet cells (fold increase of 2.2±0.5 and 1.5±0.4, respectively). (B, D) *pia* embryos treated with DMSO or DAPT from 25 to 96hpf are devoid or secretory cells in the gastrointestinal tract. P, pancreas. Scale bars : 50 µM

Supplementary Figure Legends :

Figure S1: *ascl1a* is expressed in the subpopulation of the intestine.

Ventral views with anterior to the left of 55 hpf embryos analyzed by double fluorescent WISH with *foxA3* and *ascl1a* probes combined with cell nuclei staining by the far-red fluorescent DNA dye *DRAQ7*™. Confocal sections showing that the intestinal bulb (ib) labeled by the endodermal marker *foxA3* (in red) displays a scattered distribution of *ascl1a*-expressing cells (in green). L: liver, Ib: intestinal bulbe, S: somites. Scale bars : 50 µM

Figure S2: the *ascl1a/pia* mutants do not display any apparent defects in the formation of the enterocytes.

Confocal sections of ventral views of embryos with the anterior to the left. The general morphology of the digestive tract of the *pia* mutant does not appear disturbed as revealed by the TO-PRO-3 iodide labeling of the cell nuclei at 72 hpf (A-B). Immunohistochemistry with $4^{E}8$ antibody shows that the brush border of the enterocytes seems to be correctly formed in the *pia* mutant at 5 dpf (C-D). Scale bars : 50 µM

Figure S3: Ascl1a is a master regulator upstream of *neurod1, pax6b*, *nkx2.2* and *ngn3*. Ventral views with anterior to the left of 72 hpf embryos analyzed by fluorescent (A-F) or visible (G-H) WISH. **(A-F)** Confocal projections showing that all the gastrointestinal cells expressing *neurod1, pax6b* or *nkx2.2* are absent in the *pia* mutant whereas the pancreatic expression of these factors is not affected. **(G-H)** *ngn3* is expressed in few scattered cells (arrow) of the gastrointestinal tract of wild-type embryos (G) but is not detected in the *pia* mutant (H). Scale bars : 50 µM

Figure 1

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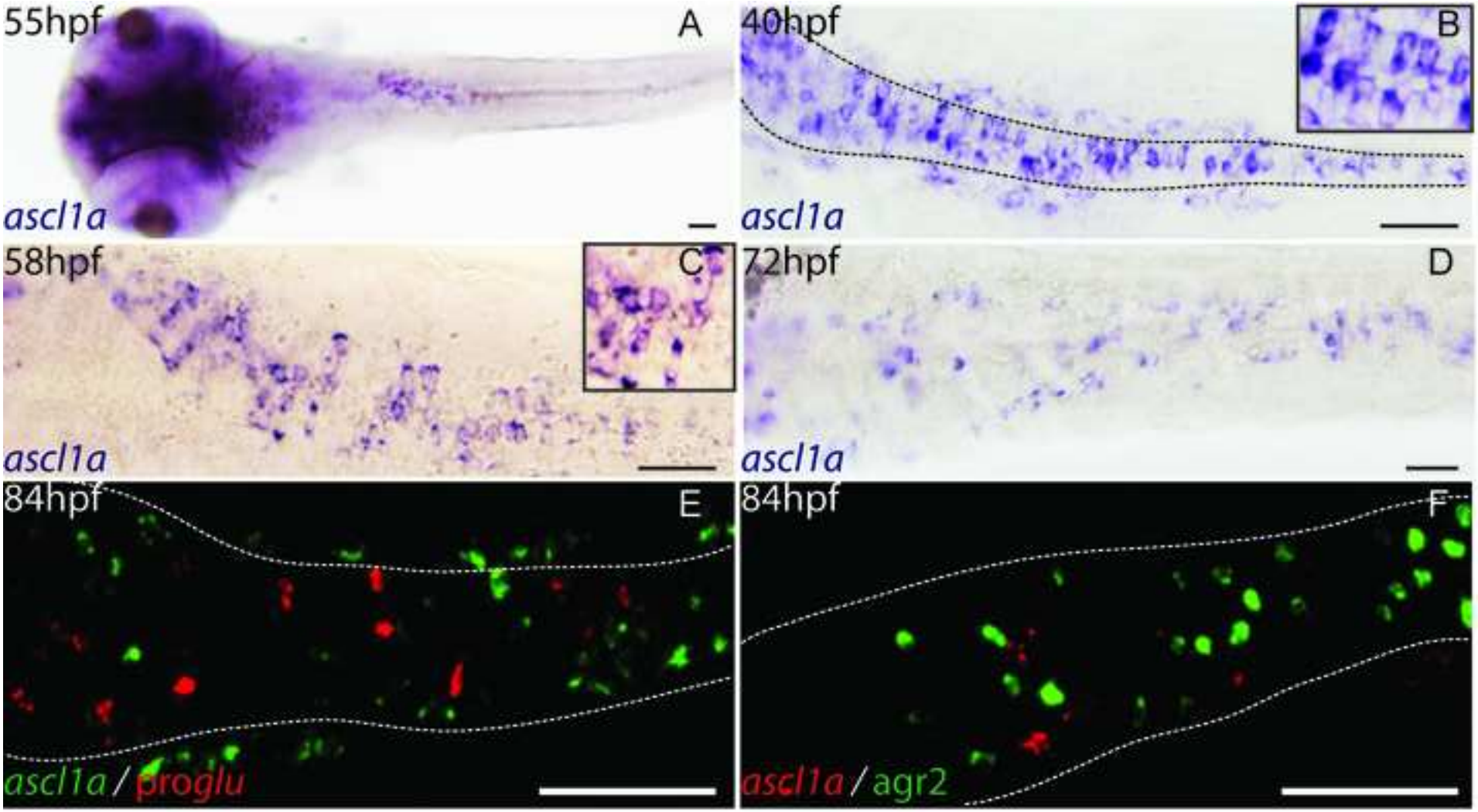

Figure 2

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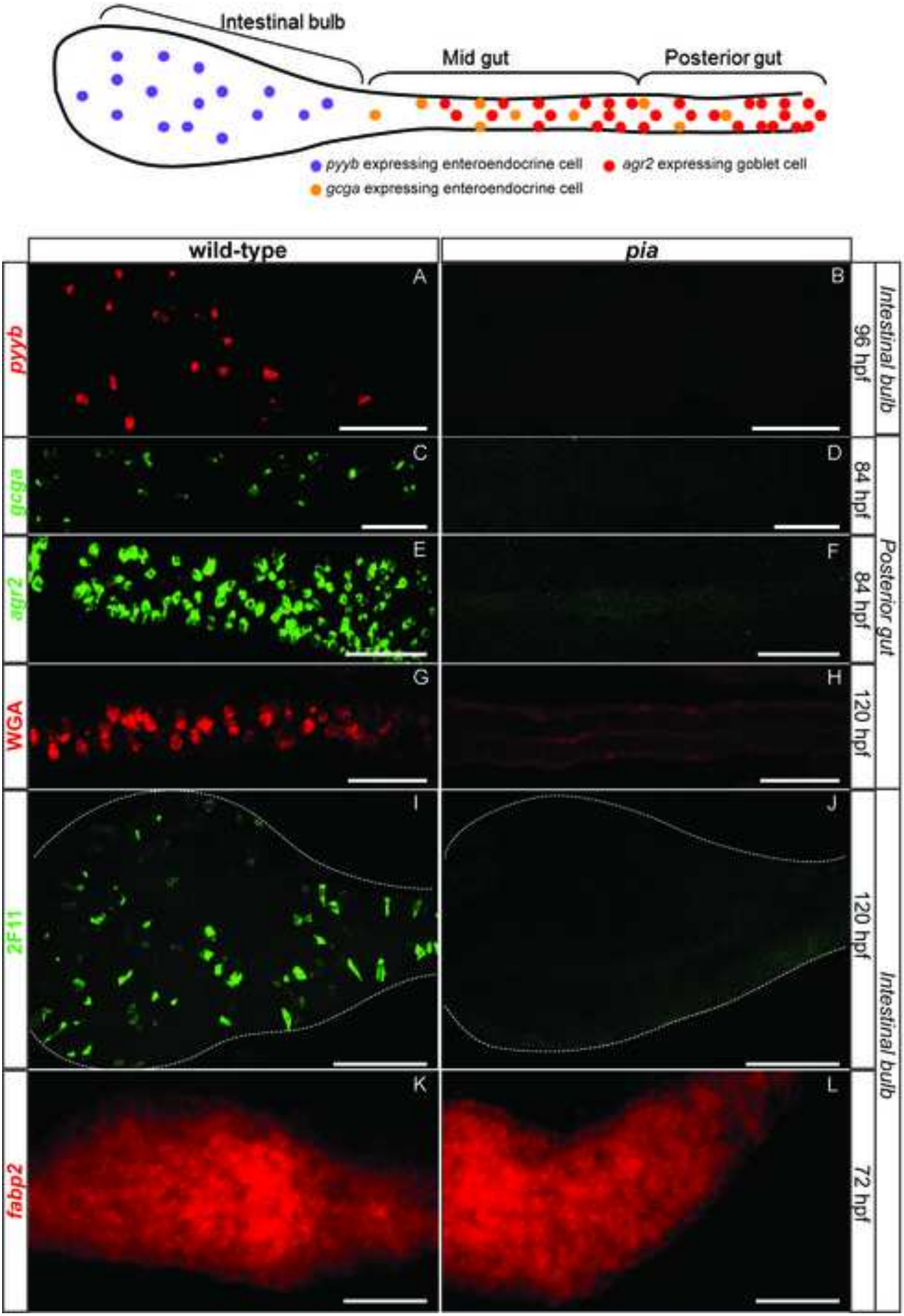

Figure 3

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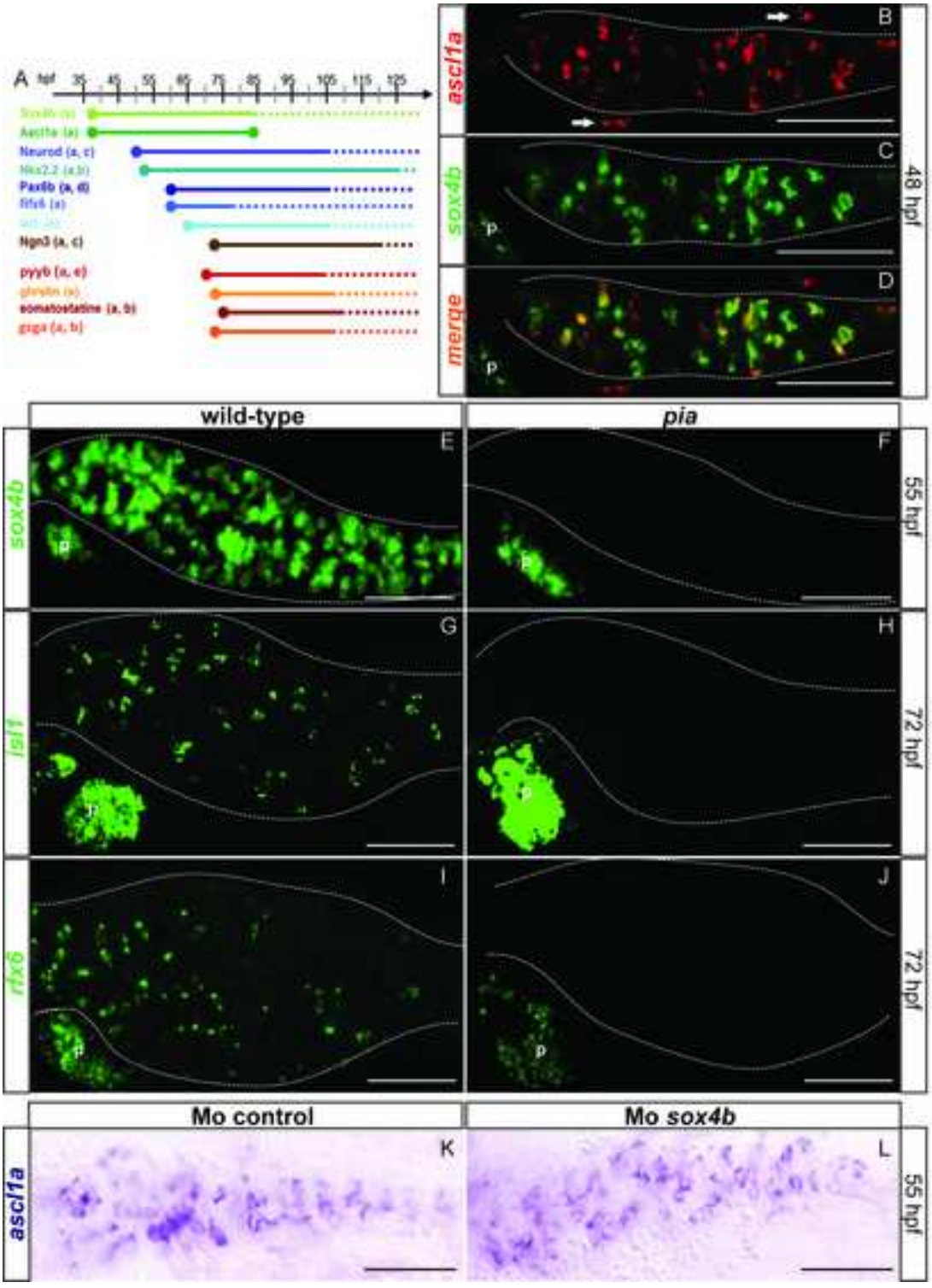

Figure 4

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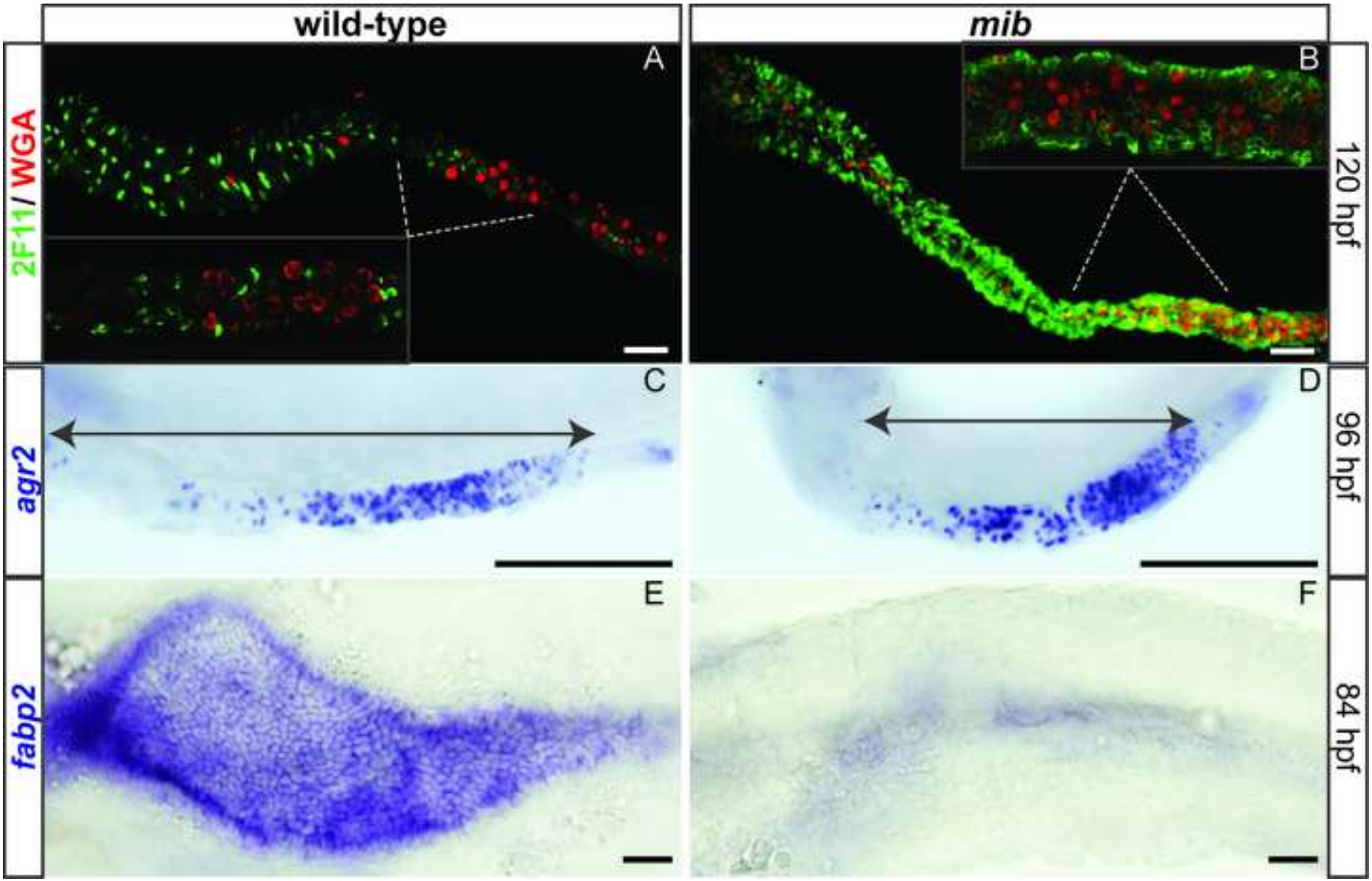

Figure 5

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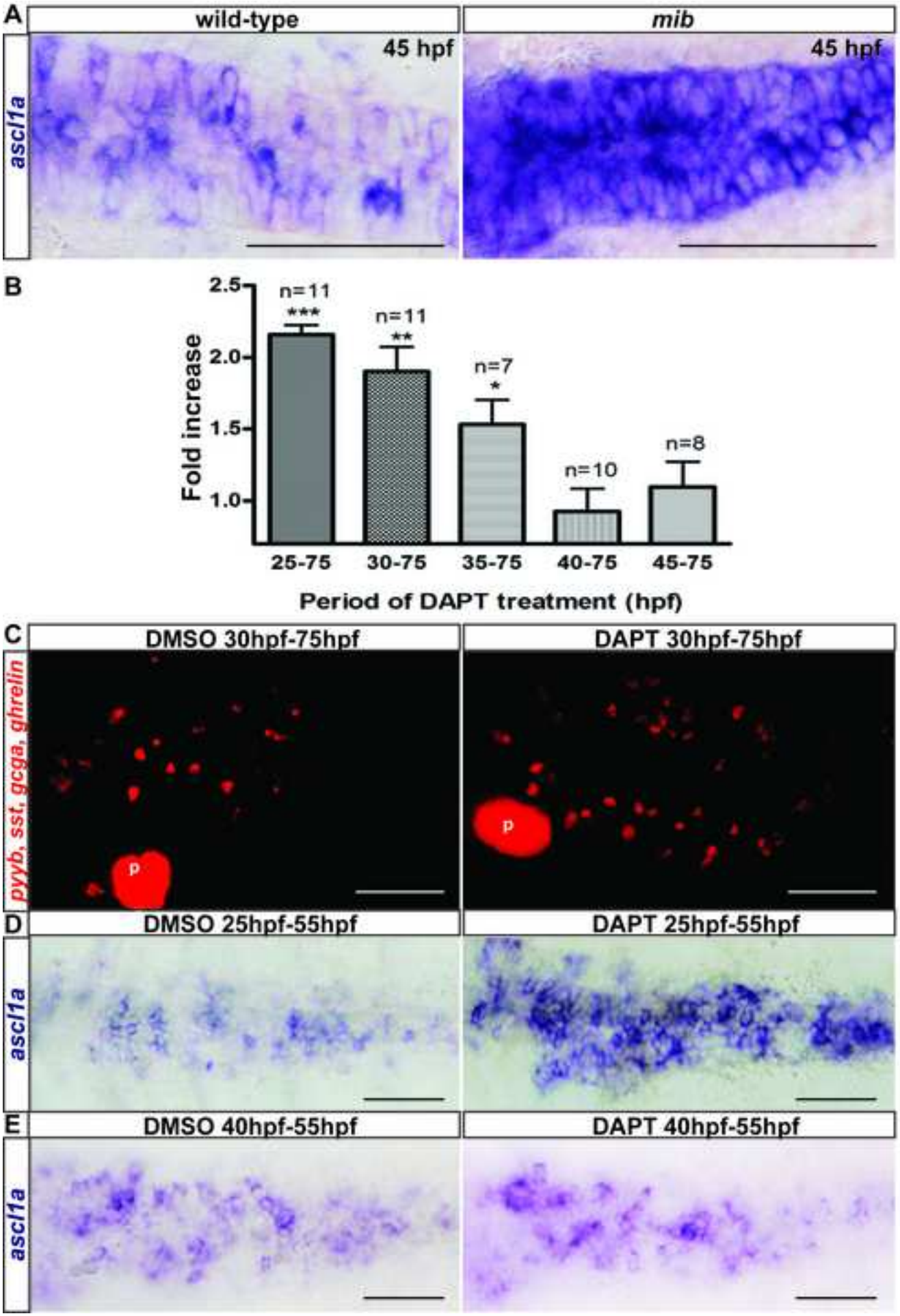

Figure 6

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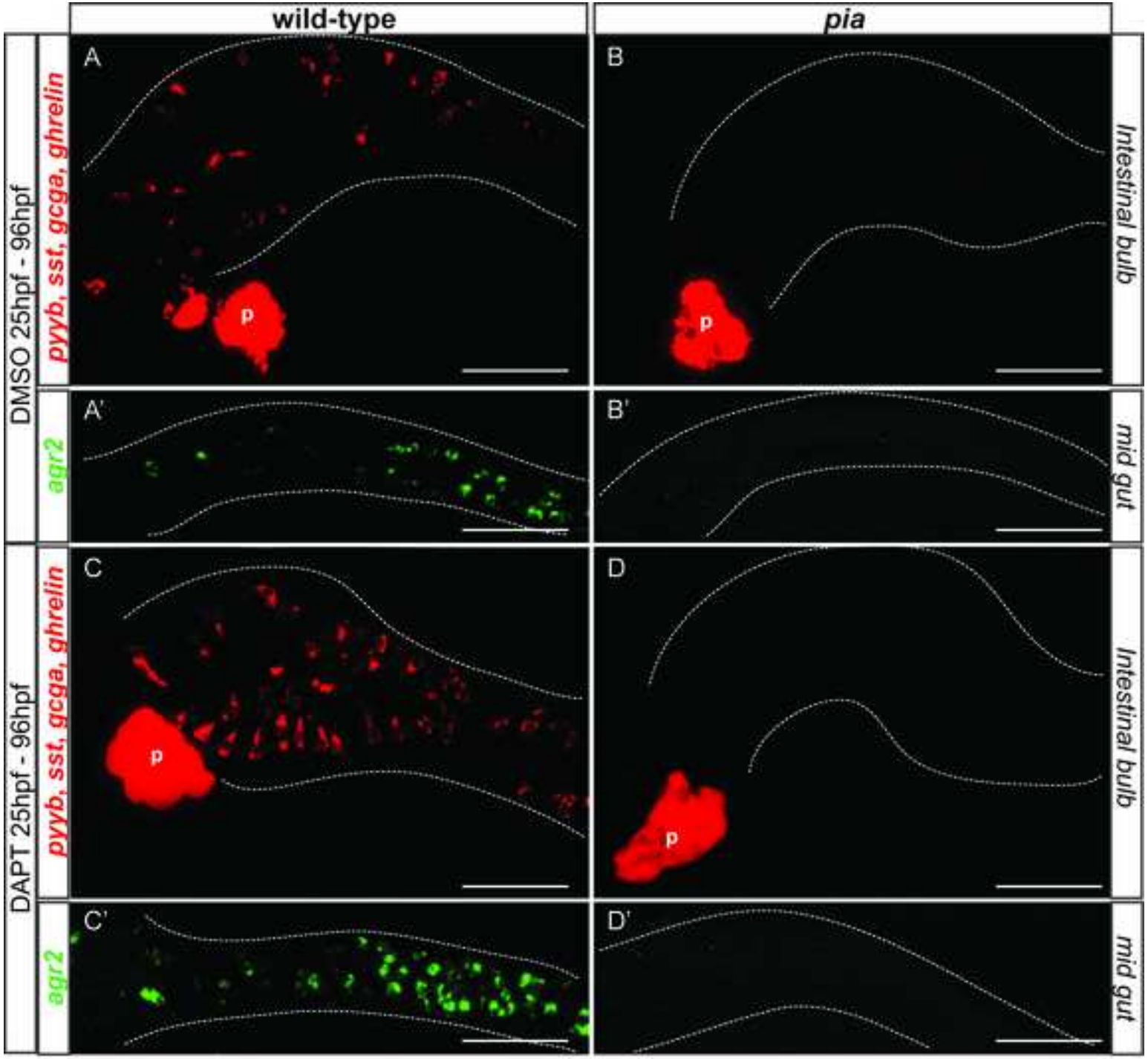

Figure S1

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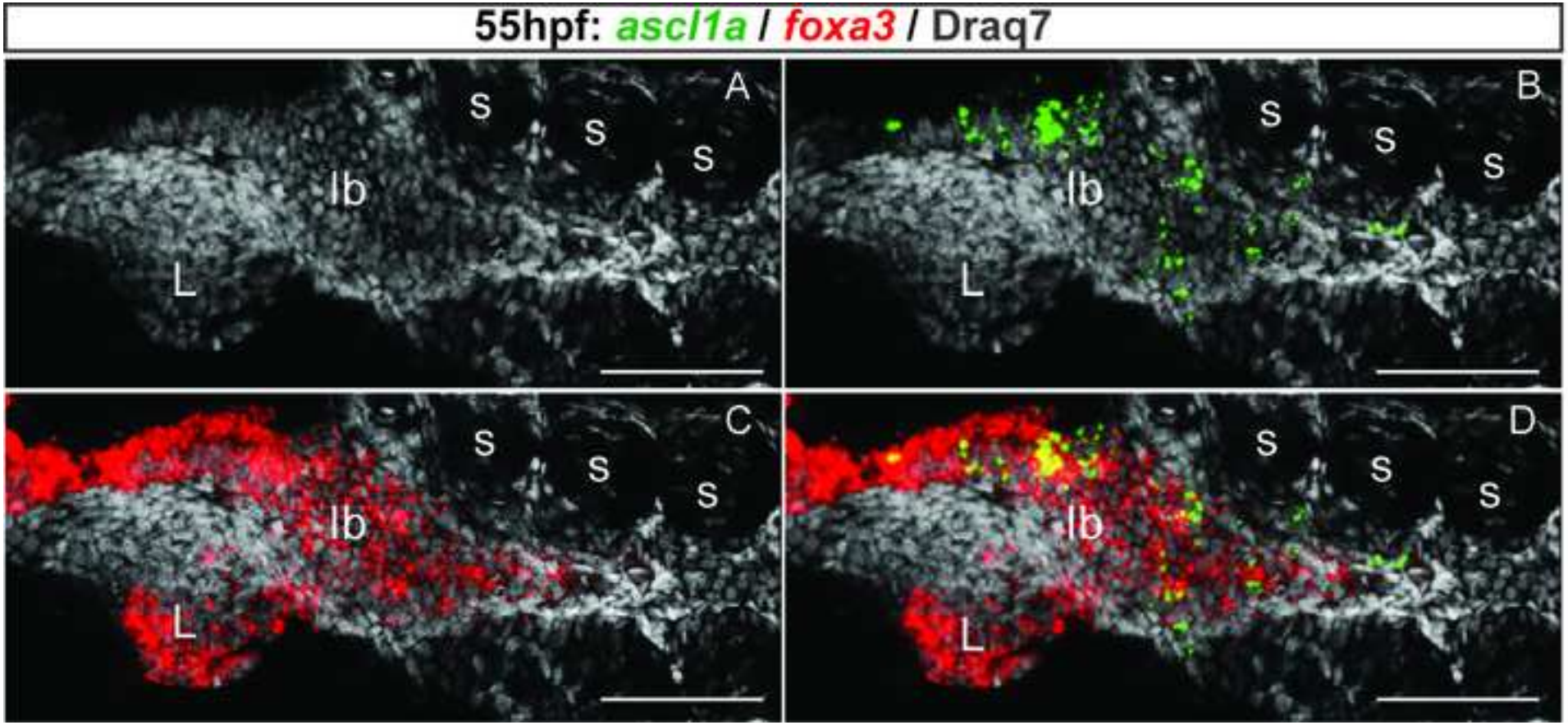

Figure S2

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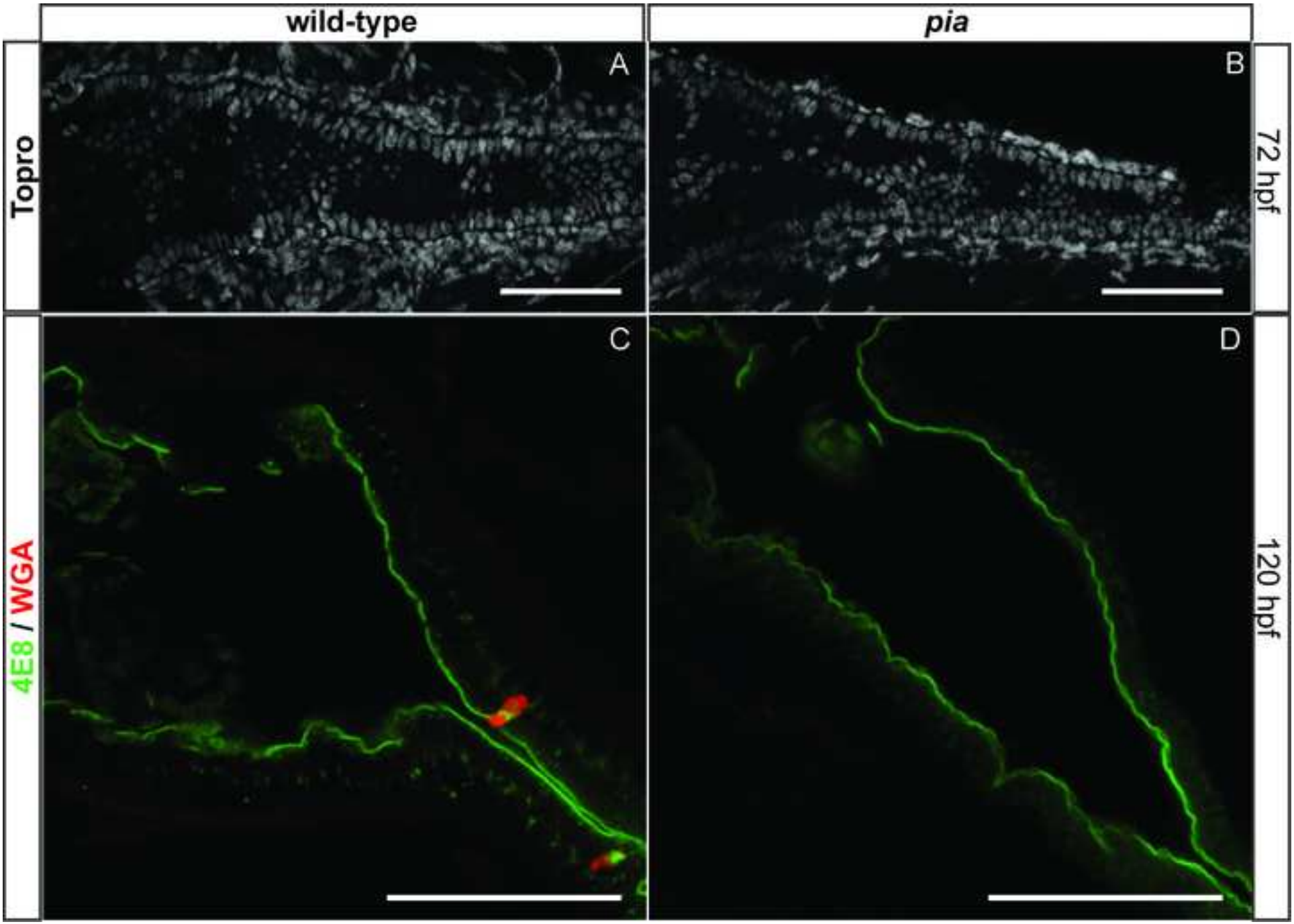

Figure S3

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