Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine

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Summary

The transparency of the juvenile zebrafish and its genetic advantages make it an attractive model for study of cell turnover in the gut. BrdU labelling shows that the gut epithelium is renewed in essentially the same way as in mammals: the villi are lined with non-dividing differentiated cells, while cell division is confined to the intervillus pockets. New cells produced in the pockets take about 4 days to migrate out to the tips of the villi, where they die. We have generated monoclonal antibodies to identify the absorptive and secretory cells in the epithelium, and we have used these antibodies to examine the part that Delta-Notch signalling plays in producing the diversity of intestinal cell types. Several Notch receptors and ligands are expressed in the gut. In particular, the Notch ligand DeltaD (Delta1 in the mouse) is expressed in cells of the secretory lineage. In an *aei* mutant, where DeltaD is defective, secretory cells are overproduced. In *mind bomb* (*mib*), where all Delta-Notch signalling is believed to be blocked, almost all the cells in the 3-day gut epithelium adopt a secretory character. Thus, secretory differentiation appears to be the default in the absence of Notch activation, and lateral inhibition mediated by Delta-Notch signalling is required to generate a balanced mixture of absorptive and secretory cells. These findings demonstrate the central role of Notch signalling in the gut stem-cell system and establish the zebrafish as a model for study of the mechanisms controlling renewal of gut epithelium.

Key words: Intestine, DeltaD, DeltaC, Notch, *mind bomb*, Zebrafish, Monoclonal antibody, Stem cells, Goblet cells, Enteroendocrine cells, Absorptive cells

Introduction

Amid the current excitement over stem cell plasticity, many basic features of normal stem cell systems in the mature vertebrate body remain obscure. This is true in particular of the lining of the gut – the most rapidly renewing of all adult tissues. Most of what we currently know about the mechanisms of intestinal cell renewal has come from studies in the mouse. In this animal, as in humans, the lining of the small intestine is organised into villi, thick fingers of tissue that protrude into the gut lumen, and crypts, thinner fingers of epithelium that extend down into the underlying connective tissue. Cell division is confined to the crypts; non-dividing differentiated cells line the villi. Four distinct classes of differentiated cells are present, all requiring continual replacement: absorptive cells (also called brush-border cells or enterocytes), mucous cells (goblet cells), enteroendocrine cells (secreting gut hormones) and Paneth cells (secreting antibacterial proteins). All of these are believed to originate from a common multipotential precursor cell type, the intestinal stem cell, located near the base of the crypts (Bjerknes and Cheng, 1999; Brittan and Wright, 2004; Cheng and Leblond, 1974; Potten, 1998; Sancho et al., 2004). Stem cells are thought to divide slowly, giving rise to new stem cells

and to committed intestinal progenitors that undergo a series of more rapid transit amplifying divisions within the crypt before finally differentiating. The differentiated cells survive only a few days (or a few weeks, in the case of Paneth cells) and are then discarded, by apoptosis and by shedding from the tips of the villi into the gut lumen.

At some poorly defined point or points in this process, descendants of the multipotential stem cell lose stem-cell potential and become restricted in their choice of final differentiated state. Studies using chemical mutagenesis in the mouse to create genetically marked clones of cells have provided some partial information about the pedigrees of the differentiated cells: one finds in the gut lining both small clones comprising a mixture of different differentiated cell types (e.g. columnar and mucous) and large clones consisting entirely of a single cell type (e.g. mucous) (Bjerknes and Cheng, 1999).

Although knowledge of the sequence and timing of the cell-fate choices remains hazy, genetic studies have identified some of the molecules that influence these decisions. Thus, activation of the Wnt signalling pathway in epithelial cells in the crypts seems to be crucial for maintenance of the proliferative stem-cell state: overactivation leads to excessive

proliferation and development of intestinal polyps (Sansom et al., 2004), while inactivation leads to a failure of crypt development (Korinek et al., 1998). BMP4 signalling acts antagonistically, inhibiting the formation of depots of stem cells in improper locations (Haramis et al., 2004; He et al., 2004).

Notch signalling, on the other hand, seems to exert control at some later step, influencing choices between alternative modes of terminal differentiation in the digestive tract. In the embryonic pancreas, disruption of the genes coding for Delta1 or the Notch pathway effectors RBP-Jκ or Hes-1 leads to precocious and increased production of pancreatic endocrine cells (Apelqvist et al., 1999; Jensen et al., 2000), whereas loss of Hes-1 in the intestine causes an increase in the proportion of both enteroendocrine and goblet cells (Jensen et al., 2000). Mice with knockout mutations in *Math1*, which codes for a bHLH transcription factor that is indirectly regulated by Notch in other tissues, show a loss of all secretory cells, including goblet, enteroendocrine and Paneth cells (Yang et al., 2001). Conversely, inhibitors of the γ-secretase that is required for activation of Notch (but also for cleavage of many other transmembrane proteins) have been found to cause overproduction of secretory cells in the adult mouse gut (Milano et al., 2004; Wong et al., 2004).

Yet another signalling system, via ephrin B1 and its receptors EphB2 and EphB3 (Batlle et al., 2002), governs migration and sorting of the differentiated cell types, whereby the absorptive, enteroendocrine and mucous cells migrate upwards to cover the intestinal villi, while the Paneth and stem cells remain at the bottom of the crypts.

Despite the increasing amount of information about these molecular mechanisms, we still do not know how they are coordinated with one another, at what precise points in the gut cell lineages they act or how they couple the progress of differentiation to changes in proliferative behaviour in the population of committed progenitors as they go through their transit amplifying divisions. In this paper, as a first step towards addressing these issues, we turn from the mouse to the zebrafish and examine the part that Notch signalling plays in controlling the ratio of secretory to absorptive cell types in its gut epithelium.

The juvenile zebrafish has many advantages for such investigations: it is transparent, so that the living gut cells can be observed through the body wall (Fig. 1A); a good collection of Notch pathway mutants is available; and techniques for transgenesis and gene misexpression are well developed. The embryonic and early larval stages of zebrafish gut development have been well described (Field et al., 2003a; Field et al., 2003b; Ober et al., 2003; Wallace et al., 2005; Wallace and Pack, 2003). To use the zebrafish as a model for gut renewal in adult mammals, however, we first have to show that the process in zebrafish occurs in essentially the same way as in mammals, and we have to have reagents for the zebrafish that will allow us to monitor production of the different gut cell types. In this paper, we demonstrate that the pattern of epithelial renewal in the zebrafish is indeed similar to that seen in mammals. We present a panel of monoclonal antibodies that recognize the different gut cell types. And we use these antibodies to show that in mutant fish that are null for the Notch ligand DeltaD [encoded by the *after eight* (*aei*; *dld* – Zebrafish Information Network) gene (Holley et al., 2000)], secretory

cells are produced in excess, while in the *mind bomb* (*mib*) mutant, where all Delta-Notch signalling is blocked (Itoh et al., 2003), almost all the cells of the gut epithelium adopt a secretory character. Thus, it seems that Notch signalling does not merely influence cell fate decisions, but is essential to prevent the gut cells from all differentiating in the same way.

Materials and methods

Animals
Zebrafish were raised according to standard procedures (Westerfield, 1993) at 28°C on a 14- to 10-hour light-dark cycle. Delta1$^{LacZ/+}$ mice were as described by (Morrison et al., 1999).

Histology
Embryos and larvae were fixed overnight in a 4% formalin solution in phosphate-buffered saline (PBS) at 4°C; fixative penetration was ensured by removing the head, tail and skin of the larvae. Fixed tissue was embedded in agar and cryosectioned as described by (Haddon and Lewis, 1996).

For plastic sections, tissue was fixed in a 4% formalin/2.5% glutaraldehyde mixture, embedded in araldite, sectioned at 1-2 μm and counterstained with Toluidine Blue.

Mouse tissue was processed for β-galactosidase detection (Morrison et al., 1999).

Proliferation analysis
Sections were boiled in 10 mM citric acid for 5 minutes, cooled at room temperature for 20 minutes and rinsed in PBS before immunolabelling overnight at 4°C with anti-PCNA antibody (1:200, Lab Vision). After further rinses, a rabbit anti-mouse-FITC secondary (1:50, DAKO) was applied and rinsed before counterstaining with TOPRO-3 and mounting in SlowFade (Molecular Probes).

For time-course experiments, 1-month-old fish were starved for 24 hours, then bathed for 2 hours [between ZT6.5 and ZT8.5 (Dekens et al., 2003)] in a 0.5% BrdU solution, briefly rinsed and kept for a further 0, 24, 48, 72 or 96 hours before fixation for cryosectioning as described above. Sections were incubated for 10 minutes in 1% H_2O_2 in methanol, rinsed in PBS, hydrolysed for 10 minutes in 2% HCl, neutralised for 10 minutes in 0.1 M sodium borate, rinsed, blocked for 1 hour in 10% sheep serum, and immunostained with mouse anti-BrdU antibody (1:50, DAKO) and a rabbit anti-mouse-FITC secondary (1:50, DAKO). Nuclei were counterstained with TOPRO-3 (Molecular Probes).

TUNEL analysis
TUNEL analysis was performed using the In situ Cell Death Detection kit (Roche). Sections were boiled for 1 minute in 10 mM citric acid, rinsed in PBS and incubated in Tris-HCl 0.1 M, pH 7.5, 3% BSA, 20% sheep serum for 30 minutes at room temperature before processing according to the manufacturer's instructions.

Monoclonal antibody production and screening
The entire digestive system was removed from adult fish and washed in PBS containing Complete EDTA-free Protease Inhibitor (Roche). The tissue was crudely chopped and then homogenised by repeated pipetting in a solubilisation buffer containing 1% Igepal CA630 (Sigma), 10 mM Tris:HCl (pH 7.4), 150 mM NaCl and complete EDTA-free protease inhibitors. The preparation was cooled on ice for 20 minutes; nuclei and insoluble material were then pelleted in a microfuge, and the lysate aliquoted and stored frozen until use.

Four 6-week-old BALB/c mice were immunised by repeated subcutaneous injections with a 50:50 mixture of gut lysate with complete Freund's adjuvant (initially) and incomplete adjuvant (subsequently). A mouse generating good immune responses was boosted with a final intra-peritoneal injection and then killed 4 days

later. The spleen was removed and splenocytes were fused to the mouse SPII myeloma cell line using standard procedures and plated over nine 96-well plates. Ten days later the hybridoma supernatants were screened by immunohistochemical staining of formalin-fixed gut sections and hybridomas secreting antibodies labelling discrete cell types or structures were cloned and expanded. Antibodies were affinity-purified on a protein A sepharose column.

Western blots

Fish gut lysate was run on NuPAGE 4-12% Bis-Tris Gel (Invitrogen) for 35 minutes at 200 V under non-reducing conditions, using the XCell SureLock mini-cell system (Invitrogen). Gels were blotted onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences), overnight at 4°C using the XCell II blot module (Invitrogen). Membranes were blocked in 5% Marvel in PBST for 2 hours, incubated with the appropriate primary antibodies (1:500 in PBST/1% Marvel) for 1 hour at room temperature, washed and imaged by chemiluminescence using HRP-tagged anti-mouse-IgG antibody from the ECL Plus Western Blotting Reagent Pack and Detection System (Amersham Biosciences), recorded on MXB films (Kodak) which were developed using an XOMAT processor (Kodak).

Isotyping

Antibodies were isotyped using the Immunotype Mouse Monoclonal Antibody Isotyping kit (Sigma).

Immunolabelling

Cryosectioned tissue was immunolabelled for 2 hours with antibodies 2F11, 2H9, 3G12, 4B7/1, 4B7/2, 4E8, 5F11 or 6G5 all diluted to ~10 µg/ml in 10% goat serum, 2% bovine serum albumin, 0.1% Triton, 10 mM sodium azide in PBS. Sections were rinsed in PBS before incubation with rabbit anti-mouse-FITC secondary (1:50, DAKO). Sections were rinsed in PBS before nuclear counterstaining with TOPRO-3 and mounting in SlowFade (Molecular Probes).

For double immunolabellings, we used mouse anti-DeltaD (10 µg/ml) (Itoh et al., 2003), rabbit anti-laminin (1:25, Sigma) and goat anti-rabbit-Alexa488 (1:250, Molecular Probes). 2F11 was biotinylated using the EZ-link™ NHS-biotin (Pierce) at a molar ratio of 1:40 (antibody:NHS-biotin) followed by streptavidin-Alexa488 (1:75, Molecular Probes) for detection. Actin was stained with phalloidin-Alexa488 (1:75, Molecular Probes) and mucus by wheat-germ agglutinin-Alexa488 (1:100, Molecular Probes). Sections were analysed on a Zeiss LSM510 confocal microscope.

Immunogold labelling and electron microscopy followed standard procedures (Slot and Geuze, 1985; Tokuyasu, 1986).

RNA preparation and RT-PCR

Total RNAs were prepared from the middle and posterior segments of juvenile zebrafish gut using the SV Total RNA Isolation System (Promega). Reverse transcription was carried out using the RETROscript kit (Ambion). Details of the Notch, Delta and Serrate primers used for RT-PCR are available on request.

Results

The adult zebrafish digestive tract, like that of mammals, is specialized for different functions in different regions. There is no discrete stomach, but the first third of the gut tube caudal to the oesophagus is enlarged to form a food-storage compartment known as the intestinal bulb (Pack et al., 1996; Rombout, 1977). Electron-microscope studies in other cyprinid fish have distinguished three regions with distinct absorptive functions. The first (region I), which represents 65 to 75% of the intestine and includes the intestinal bulb, is involved in lipid absorption; the second (region II) represents 25-35% of the

intestine and is a protein absorptive zone; finally, the third (region III), which represents less than 5% of the intestine, is specialized for ion transport and water absorption, like the colon of mammals (Rombout, 1977; Stroband and Debets, 1978).

The earliest stages of zebrafish gut development, up to three days post fertilization (dpf) have already been described (Field et al., 2003a; Field et al., 2003b; Ober et al., 2003; Wallace and Pack, 2003). Here, we focus on the later period, up to 1 month after fertilization, by which time the gut has essentially attained its mature fully differentiated structure (Fig. 1B).

At 72 hours, most of the components of the digestive tract, including the liver and pancreas, have formed. The intestine appears as a narrow tube bent to the left in its anterior part and with an enlargement at the level of the intestinal bulb (Mayer and Fishman, 2003; Wallace and Pack, 2003). The connection between the pharynx and the future oesophagus has not yet opened, and the anus is not yet patent. The anus becomes patent at 96 hours (Wallace and Pack, 2003) and by this stage the whole length of the gut is innervated (Shepherd et al., 2004).

Larvae start autonomous feeding at 5 days, but the intestine is still very immature, consisting of a simple tube extending from mouth to anus, which is now open to the exterior. The swim bladder, liver, gall bladder and pancreas are now all present as substantial (and functional) organs connected to the main gut tube.

Up to about 3 weeks of development, the digestive tube grows steadily but remains relatively straight. Over a period of a few days, between 21 and 30 days of development, the tube becomes kinked and then folded into its adult shape, with the middle region of the intestine formed into a single elongated coil (like the coil of a trumpet) as shown in Fig. 1B. We thus distinguish three anatomical segments of intestine: an anterior (A) segment, running rearward from the intestinal bulb; a middle (M) segment, directed forwards; and a posterior (P) segment running rearwards to the anus. Roughly speaking, the anterior and middle segments jointly comprise functional region I, most of the posterior segment constitutes functional region II, and the caudal extremity of the posterior segment corresponds to functional region III.

The zebrafish gut develops its mature pattern of villi and intervillous pockets progressively between 5 days and 1 month after fertilization

In cross-section at 3 days, the intestinal epithelium appears as a simple monolayered tube without villi (Fig. 1C,D). Its cells are cuboidal and not yet morphologically differentiated, although, as we describe below, they are beginning to show molecular signs of differentiation. Presence of small epithelial folds at around 5 days of development is the first obvious sign of formation of the villi (Fig. 1L). By one month of development, the entire digestive tract is lined by well-developed villi, which are longest in the intestinal bulb and decrease progressively in size towards the caudal end of the intestine (Fig. 1E,F). No crypts are present, but, as we explain below, the regions between the villi – the intervillus pockets – have a crypt-like function.

The epithelium throughout the intestinal bulb and intestine has a typical simple columnar or pseudostratified structure,

with absorptive brush-border cells and mucous goblet cells clearly visible. In electron micrographs at one month of development (Fig. 1G-J), at least three cell types are distinguishable: absorptive cells, with a brush border; goblet cells, with characteristic mucus-filled vacuoles at their apex; and enteroendocrine cells, containing darkly stained vacuoles concentrated basally.

Cells are produced in the intervillus pockets and shed from the villus tips

Despite the absence of crypts of Lieberkühn, proliferation occurs in an analogous location: the intervillus pockets. To demonstrate and analyse this pattern, we used two approaches: immunostaining for PCNA (proliferating cell nuclear antigen, DNA polymerase delta auxiliary protein), which marks cells in
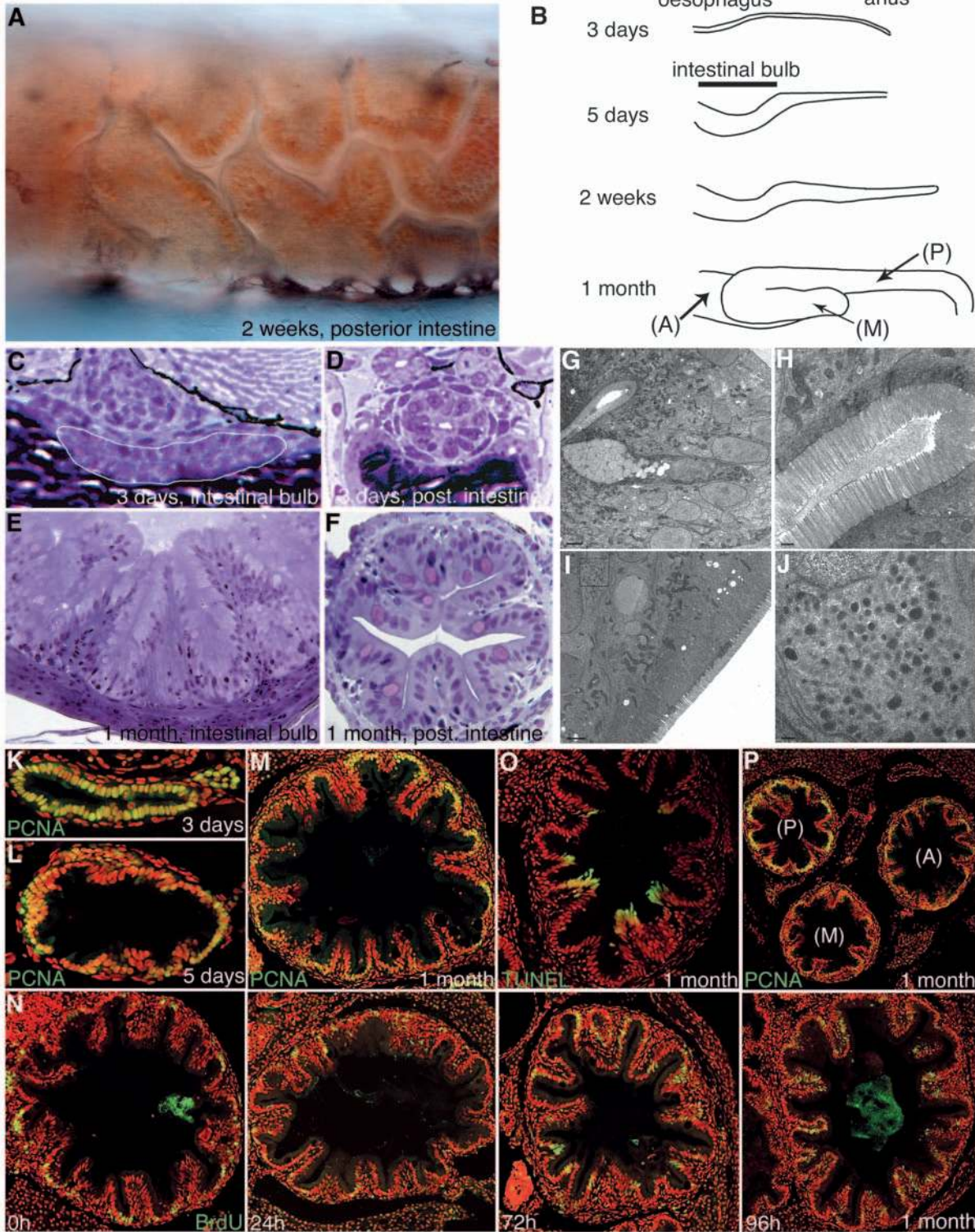
S phase as well as cells that are about to enter S phase or have recently completed it, and BrdU incorporation, which marks cells strictly in S phase.

The regionalized pattern of proliferation develops gradually as the villous structure matures. At three days post fertilisation, PCNA staining was observed in almost all the epithelial cells (Fig. 1K). However, by 5 days, cells located on the small infoldings corresponding to the future villi had a much fainter labelling, suggesting that they had stopped proliferating (Fig. 1L). By 1 month, PCNA-positive cells were only observed in the intervillus pockets, which can thus be considered as equivalent to the crypts of Lieberkühn of the mammalian intestine (Fig. 1M,P).

In mammals, the production of new cells in the crypts is associated with cell migration from the crypts onto the villi. To see whether a similar phenomenon was occurring in the 1-month-old zebrafish, we used BrdU pulse-chase labelling to trace the movements of the cells that had been in S-phase at the time of an initial BrdU pulse (Fig. 1N and Table 1). Immediately after labelling, all the BrdU-positive cells lay in the intervillus pockets. At 24 hours, labelled cells had begun to climb up the bases of the villi. At 72 hours, they lined the sides of the villi. By 96 hours they had reached the tips. These data imply that cell migration from villus base to villus tip takes about four days, a duration similar to what has been described in the mammalian intestine. Occasional BrdU-positive cells could still be seen in the intervillus pockets at 96 hours, corresponding presumably to slowly dividing stem cells or early progenitors.

The continual production and migration of intestinal cells in mammals is associated with programmed cell death as they reach the tops of the villi (Vachon et al., 2000). We examined the pattern of cell death in the zebrafish gut by TUNEL staining at 3 days and 5 days, before feeding begins, and after one month of development, all following a 24 hour fast. Scarcely any dying cells were seen at 3 or 5 days (data not shown), but at 1 month of development, there were prominent groups of TUNEL-positive cells located at the tops of the intestinal villi,

Table 1. Migration of BrdU-labelled cells from intervillus pockets towards villus tips

Time since BrdU pulse	Intervillus pockets (%)	Lower halves of villi (%)	Upper halves of villi (%)	Total BrdU-labelled cells counted
0 hours	90	10	0	32
24 hours	57	41	2	50
72 hours	27	43	30	56
96 hours	8	32	60	48

Entries give the number of BrdU-labelled cells in each region as a percentage of total BrdU-labelled cells counted. Cells were counted on six to eight villi from two labelled fish for each time-point.

presumably destined for exfoliation, especially in the A and M segments of the gut (Fig. 1O).

Generation and characterisation of new monoclonal antibodies directed against differentiated zebrafish intestinal cells

The similarities with other vertebrate intestinal epithelia show that the zebrafish gut can be an alternative model to study intestinal differentiation and renewal. However, the very limited number of molecular markers available in the zebrafish intestine is a major problem. To overcome this difficulty, we generated a panel of monoclonal antibodies directed against the zebrafish digestive tract, using a 'shotgun' approach, as described in the Materials and methods.

Supernatants from 840 hybridoma wells were screened for interesting labelling patterns on sections of intestine of 1-month-old fish. About 80% of these produced either no staining or diffuse staining that was either non-specific or restricted to the connective tissue, another relatively large group stained only goblet-cell mucus. From the remainder, we selected 11 hybridomas as sources of monoclonal antibodies for specific staining of components of the gut. All these were isotyped as IgG1 immunoglobulins. Eight of them recognised antigens in the gut epithelium and will be described here; three others, to be described elsewhere, recognised mesenchymal components in the neighbourhood of the gut tube and other specific organs of the digestive tract. The expression patterns of the eight epithelial markers were studied at 2 days, 3 days, 5 days, 2 weeks and after 1 month of development, as described below. The molecular mass of the different antigens was determined on western blots (Table 2).

Antibodies 2F11, 4B7/2 and 6G5

These three markers all appeared to label secretory cells in the one-month-old gut, but differed in their behaviour on western blots (Fig. 2A and Table 2). To compare the staining patterns on tissue sections, we biotinylated 2F11, for detection with streptavidin, and stained sections with this in combination with 4B7/2 or 6G5, which we detected with a secondary antibody. In this way, we found that all three monoclonals stain identical cell populations (Fig. 2B), at least at 5 days of development. We focused on 2F11 for further analysis.

Cells positive for 2F11 showed different morphologies as the intestine matured. At 3 days of development, they appeared rounded or cuboidal, like their neighbours in the epithelium (Fig. 5A). At 5 days, in most of the intestine, the labelled cells still had this shape, but in the intestinal bulb many had adopted

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Fig. 1. The zebrafish intestine and its pattern of cell renewal. (A) Micrograph of the posterior intestine of a living 2-week-old fish. Intestinal villi are clearly visible through the transparent body wall. The epithelial cells are coloured brown by uptake of pigment in the diet. (B) Sketches showing development of the gross anatomy of the zebrafish gut. A, anterior segment; M, middle segment; P, posterior segment. (C-F) Toluidine Blue-stained plastic sections of intestinal bulb (C,E) and posterior intestine (D,F) at 3 days and 1 month. The intestine matures rapidly from a simple tube to a more elaborate structure with villi. The intestinal bulb initially has a flattened shape (white outline in C). (G-J) Electron micrographs identify goblet cells (G), brush-border cells (H) and enteroendocrine cells (I,J). (J) Enlargement of the area boxed in the top-left corner in I, showing characteristic secretory granules. (K-M) Proliferating cells are progressively restricted to the intervillus pockets as shown by PCNA labelling (green) in the intestinal bulb. (N) Pulse-chase experiment showing the migration of BrdU-labelled cells (green) along the villi of the intestinal bulb 0, 24, 72 and 96 hours after the pulse. (O) TUNEL labelling (green) showing programmed cell death in cells at the tops of the intestinal villi. (P) At 1 month of development, proliferation is more plentiful in the posterior part of the gut, as shown by PCNA labelling (green). (K-P) Confocal optical sections; TOPRO-3 nuclear stain is in red.

Table 2. Characteristics of the intestinal antibodies

| Antibody name	Antigen size on western blot (kDa)	Expression first seen at (days)	Structures labelled
2F11	Four major bands at 170, 120, 90 and 35	3	Cytoplasm of enteroendocrine and goblet cells (but no labelling of the mucus
4B7/2	35	3	compartment). The 3 markers label identical cell types as determined by
6G5	n.d.	3	double labelling
3G12	Three major bands at 175, 120 and 70	5	Mucus compartment and cytoplasm of goblet cells, in the posterior gut mainly
4B7/1	n.d.	3	Brush border of enterocytes (apical ends of microvilli)
4E8	200	3	
2H9	170	3	Lateral membrane and basal region of epithelial cells
5F11	n.d.	2	Basement membrane

n.d., not determined.
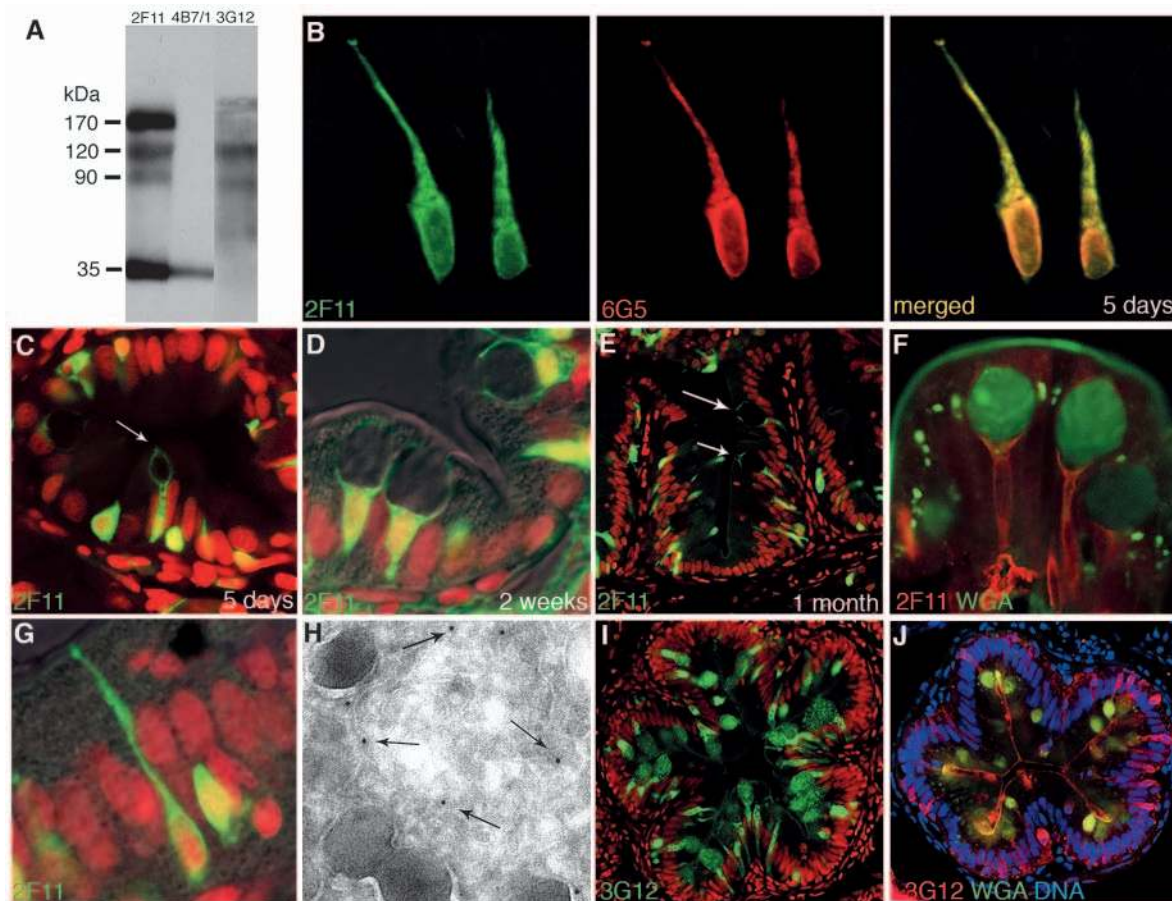
Fig. 2. Monoclonal antibodies that mark secretory cells. (A) Western blot of zebrafish gut lysate resolved under non-reducing conditions on SDS-PAGE; antibodies 2F11, 4B7/1 and 3G12 have different antigen specificities. (B) 2F11 (green) and 6G5 (red) stain identical differentiated cells in the intestinal bulb of a 5-day-old larva. (C-H) 2F11 immunolabelling. At 5 days (C), the posterior intestine contains scattered 2F11-positive cells, including a few goblet cells (arrow); goblet cells are more mature and plentiful at 2 weeks (D); by 1 month, goblet cells are also seen in the intestinal bulb (E, arrows). (F) Goblet cells in the intestinal bulb, labelled with wheat-germ agglutinin (green), which stains the mucus compartment, and 2F11 (red), which stains the rest of the cytoplasm. (G) 2F11 (green) also stains some small basal cells and cells that extend thin processes towards the gut lumen; these may be varieties of enteroendocrine cells. (H) Immunogold labelling with 2F11 (small electron-dense particles, arrows) marks the cytoplasm of an enterendocrine cell containing secretory granules (large dark vesicles). (I) 3G12 (green) stains goblet cells in the posterior intestine of a 1-month zebrafish. (J) 3G12 (red) and wheat-germ agglutinin (green) co-stain goblet cells; 3G12 stains both the mucus compartment and the rest of the cytoplasm. Nuclei are stained with TOPRO-3, shown in red (C,D,E,G,I) or blue (J).

an elongated structure with a narrow apical process extending to the gut lumen (see Fig. 4D). In the posterior intestine, a small proportion of the labelled cells had accumulations of unstained material in their apical cytoplasm, suggesting that they were

immature goblet cells (Fig. 2C). Cells of the hepatobiliary system were also labelled (data not shown).

From 2 weeks of development onwards, three categories of 2F11-positive cells could be clearly distinguished in the

Fig. 3. Monoclonal antibodies that mark brush border, epithelial cell polarity and basement membrane in the zebrafish gut. (A,B) 4E8 antibody stains the brush border, identifying absorptive cells. (A) 4E8 (red) recognises an antigen at the apices of the microvilli, distal to the actin-rich microvillus cores (green). (B) The 4E8 stain (green) is specific to absorptive cells; goblet cells (arrows) are unstained. (C-F) 2H9 (green) stains the lateral membranes and basal regions of gut epithelial cells; the staining is already visible at 3 days, and then becomes progressively stronger and more plentiful basally, especially in the villus epithelium, where cells are basally elongated. (F) Detail of the boxed area in E, showing membrane localization; basal stain may be cytoplasmic, or may reflect labelling of highly convoluted plasma membrane. (G,H) 5F11 labels the basement membrane. (G) 5F11 (red) colocalizes with laminin (green) in the intestinal epithelium at 5 days. (H) At 1 month, 5F11 (green) staining outlines the intestinal epithelium. (B-F,H) TOPRO-3 nuclear stain is shown in red.
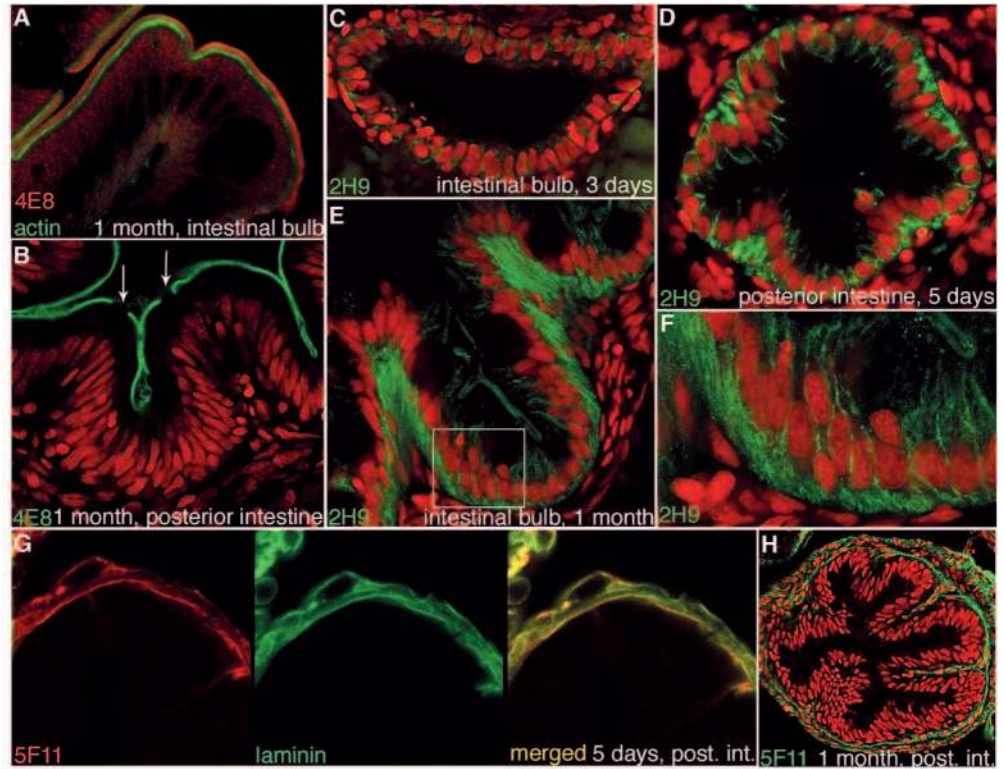
intestinal epithelium. Cells with their apical cytoplasm distended with 2F11-negative material were clearly goblet cells; at two weeks, they were observed mainly in the posterior intestine (Fig. 2D), but by one month of development they were also seen in the anterior gut (Fig. 2E). Double labelling with 2F11 and wheat-germ agglutinin, which binds N-acetylglucosamine present in intestinal mucus, confirmed that 2F11 identified all goblet cells, staining their cytoplasm but not their mucus storage compartment (Fig. 2F).

A second category of 2F11-positive cells had a basally located body and nucleus and a very narrow extension towards the lumen of the gut (Fig. 2G). These were seen all along the intestine. Finally, 2F11 also labelled some small cells located near the basement membrane and lacking apical extensions (Fig. 2G). It is probable that these two categories represent different varieties of enteroendocrine cells (Rombout, 1977), although some of them may be goblet cell precursors. To check that enteroendocrine cells are indeed labelled with 2F11, we used transmission electron microscopy: cells clearly identifiable as enteroendocrine by virtue of their secretory granules were reproducibly labelled with 2F11 immunogold particles (Fig. 2H), whereas brush-border (absorptive) cells were unlabelled. We also saw a class of cells with dark cytoplasm that were 2F11 positive but contained no apparent secretory granules. They were not typical absorptive cells, and could have been goblet or enteroendocrine precursor cells. Taking all this evidence together, we suspect that 2F11 is a marker of all gut secretory cells, both mucous and enteroendocrine, corresponding to the class of cells that are lost in *Math1* mutants in the mouse (Yang et al., 2001).

Antibody 3G12
Antibody 3G12 marks goblet cells specifically, but not all goblet cells. Unlike 2F11, it labels both the mucus compartment and other parts of the cytoplasm. On western blots, 3G12 stains diffuse bands, consistent with a variably glycosylated antigen or set of antigens (Fig. 2A).

3G12-labelled cells first became visible in small numbers (one or two cells per section) at 5 days of development. They were concentrated in the posterior gut, where they had become very numerous by one month (Fig. 2I); here, the 3G12 pattern appeared to be the same as the wheat-germ agglutinin pattern (Fig. 2J). However, goblet cells present more anteriorly, in the A and M segments of the intestine, including the intestinal bulb, were generally not labelled with 3G12, although clearly stained with wheat-germ agglutinin; occasional cells in these regions showed spots of 3G12 labelling but lacked any mucus compartment. Thus, 3G12 is a marker for mucus-secreting cells in the posterior gut, and in the A and M regions also recognises a minority cell type that we cannot yet categorize.

Antibodies 4B7/1 and 4E8
Co-labelling with phalloidin, which reacts with the actin of the microvilli, showed that 4B7/1 and 4E8 label material located just above the actin-rich area of the brush border – possibly some component(s) of the glycocalyx of the absorptive cells (Fig. 3A). Neither of these antibodies reacted with goblet cells, which showed up as gaps in the staining pattern (Fig. 3B). Thus, 4B7/1 and 4E8 can be used as markers for brush-border cells.

Antibody 2H9
Antibody 2H9 recognises a membrane-associated epitope on the gut epithelial cells (Fig. 3C-F). At 3 days of development, staining is seen throughout the epithelial population, where it is concentrated basolaterally but is faint. By 5 days, staining

Fig. 4. Secretory cells express the Notch ligand Delta and their numbers are increased in aei^{AR33}, a mutant lacking DeltaD. (A) Intestine of adult mouse heterozygous for a *lacZ* knock-in at the *Delta1* (*Dll1*) locus. β-Galactosidase activity (blue) is a reporter for present or past expression of *Delta1* expression; the stain is seen in scattered cells, many of which can be clearly identified as goblet cells. (B) Expression of Notch pathway components in zebrafish gut, analysed by RT-PCR. *deltaC* and *deltaD* are strongly expressed in the gut, while *deltaA* and *deltaB* are not. (C) DeltaD (zdd-2 immunostain in green, arrows) is visible in a subset of secretory cells (2F11, red) in the wild-type zebrafish intestine at 4 days. (D,E) Sections of intestinal bulb of 5-day-old wild-type larvae (D) and aei^{AR33} homozygotes (E) stained with 2F11; TOPRO-3 nuclear stain is red. (F) The proportion of 2F11-positive cells is increased all along the length of the gut in aei^{AR33} when compared with wild type; data points show mean and s.e.m. from counts of sections of 12 larvae of each genotype. (G) Total numbers of epithelial cells per section for the same set of specimens.
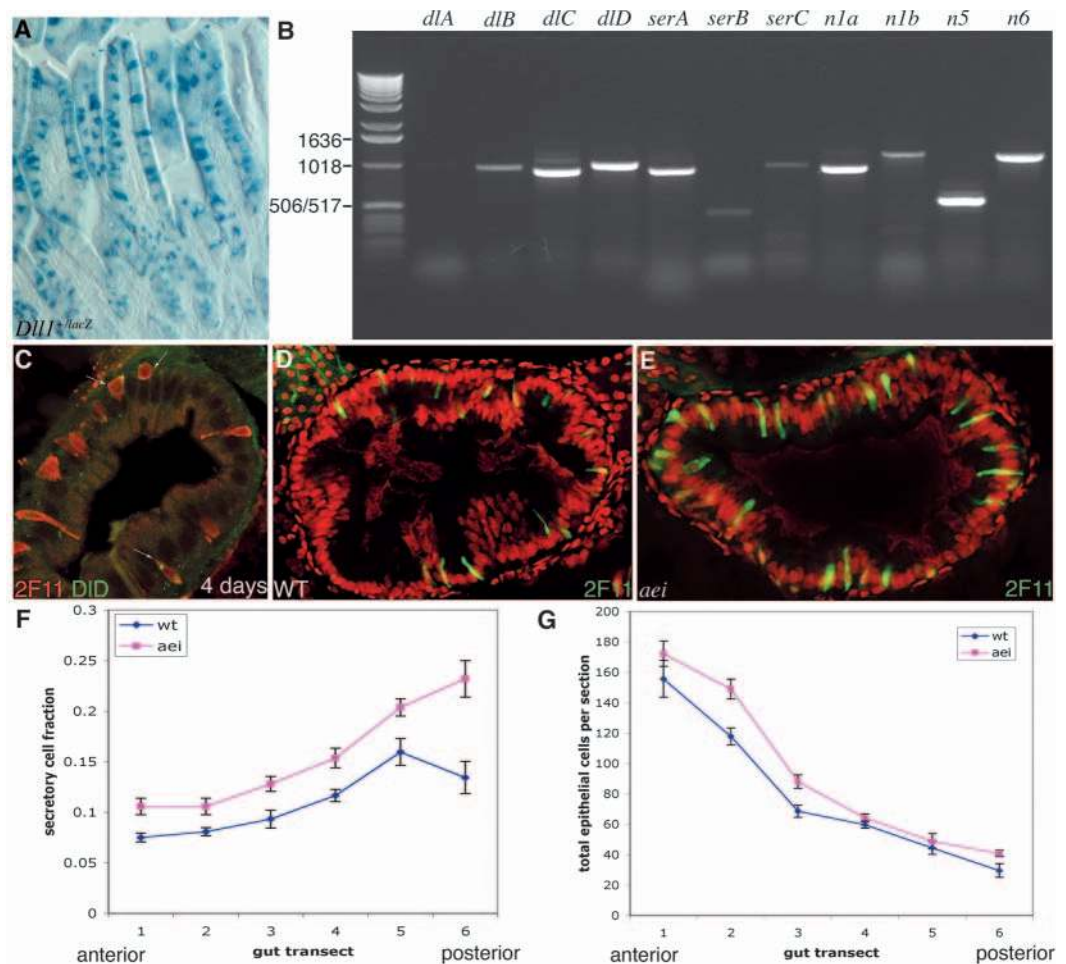
was stronger, and by one month it appeared especially prominent in the basal parts of the elongated cells of the villi. Staining in the intervillus pockets was weaker, but still concentrated basally. Although the basal staining seemed diffuse and cytoplasmic in some cases, we suspect that this was because the plasma membranes of the cells were extremely convoluted and interdigitated basally (TEM data not shown). 2H9 is potentially a useful marker of apicobasal polarity in the epithelium.

Antibody 5F11

5F11 stained the outline of the intestinal epithelium at an earlier stage than any of our other antibodies; this staining was visible already after 2 days of development and remained strong at subsequent stages. Colocalisation with laminin in doubly stained sections indicated that 5F11 is a marker for the basement membrane (Fig. 3G,H).

Gut cells expressing the Notch ligand Delta have a secretory fate

Our focus of attention in the zebrafish gut was guided by our previous observations on mice heterozygous for a *lacZ* knock-in mutation of the delta 1 (*Dll1*) locus. In these mice, β-galactosidase activity serves as a reporter of the normal

expression pattern of *Dll1* (Beckers et al., 1999; Morrison et al., 1999). Because β-galactosidase perdures, cells that are positive for this marker include both those that are currently expressing *Dll1* and those that expressed *Dll1* at some time in the past, up to ~1 day previously in the embryo (Morrison et al., 1999), and perhaps more than 1 day in the adult. In sections of adult intestine, the β-gal-positive cells are scattered in the epithelium of the villi, where many of them are clearly identifiable as goblet cells (Fig. 4A). They are visible also in the crypts, where Notch1 is known to be expressed (Schroder and Gossler, 2002), although here the staining is less intense and there is less marked contrast between stained and unstained cells. All this strongly hints that Delta-Notch signalling plays a part in singling out the goblet cells, and perhaps other minority cell types, for a fate different from that of their neighbours.

To see which Notch ligands and receptors are normally expressed in the gut of the zebrafish, we extracted total mRNA from the M and P segments of the juvenile gut (including both epithelium and adjacent mesenchyme), and performed RT-PCR. In this way, we found (Fig. 4B) that *deltaC*, *deltaD* and *jagged 1b/serrateA* were all clearly expressed, as were *notch1a*, *notch5* and *notch6*; *deltaB*, *jagged 2/serrateB*, *jagged 1a/serrateC* and *notch1b* produced only weak bands; and

Fig. 5. Intestinal phenotype of mib^{ta52b} homozygotes. (A,B) 2F11 staining in the intestinal bulb, comparing three representative pairs of wild-type (A) and mib^{ta52b} (B) specimens at 3 days. The vast majority of epithelial cells are switched to a secretory fate in mib^{ta52b}. (C,D) At the same time, the number of cells expressing DeltaD (zdd-2 antibody, green, indicated by arrows in C) is greatly increased in the mutant. Membrane localization of DeltaD in mib^{ta52b} reflects the failure of Mib-dependent Delta internalization. (E,F) 4E8 staining (green) shows deficit of absorptive cells in three-day mib^{ta52b}. (G,H) 2H9 staining (green) shows abnormal epithelial cell polarity in mib^{ta52b}. (I,J) Staining of the basement membrane with 5F11 (green) is also fainter in mib^{ta52b} than in wild-type. (K,L) At 4 days, many dying cells are seen in mib^{ta52b} (L) but not in wild type (K). (A-L) TOPRO-3 nuclear stain is red.
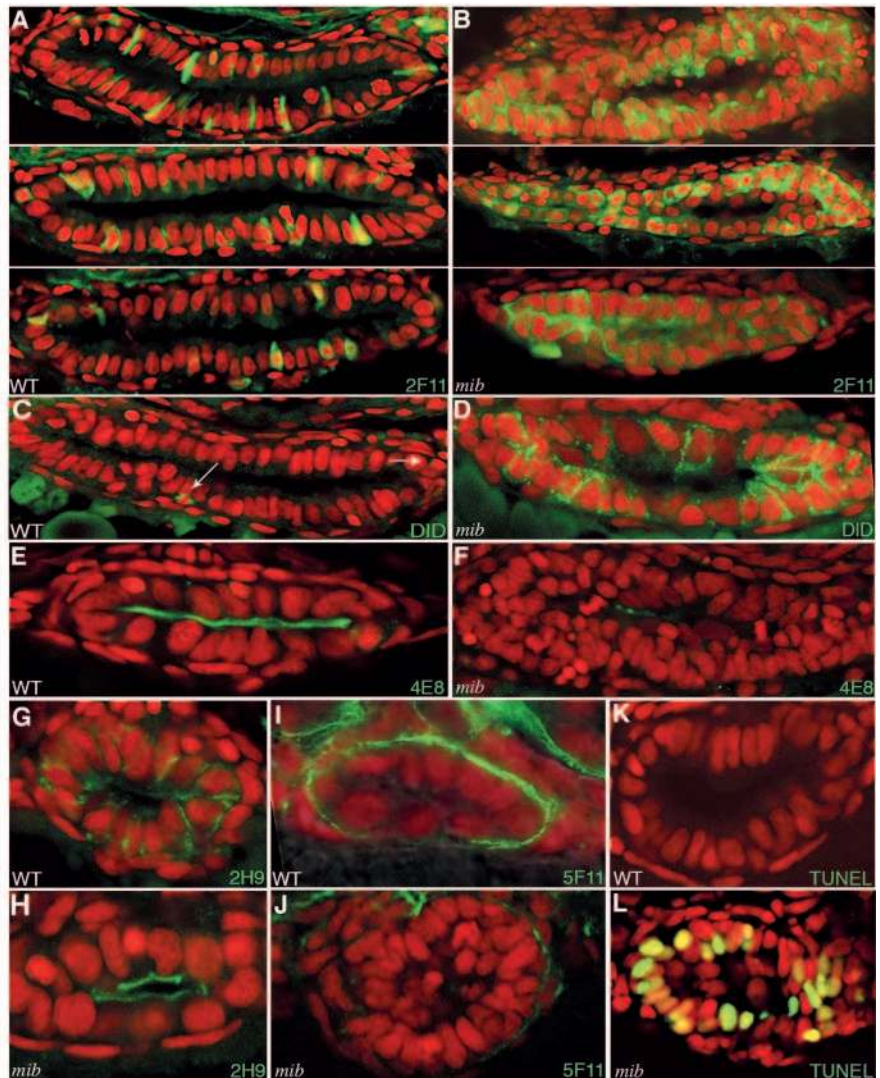
deltaA was undetectable. Despite considerable efforts, we did not succeed with in situ hybridization in this gut tissue, but we were able to examine the expression of DeltaC and DeltaD proteins (corresponding to mammalian Delta1 and Delta4) using monoclonal antibodies. It seems probable that these are the principal Notch ligands expressed in the gut epithelium (Schroder and Gossler, 2002). We have focused on two mutations that disrupt these genes or their activity: aei^{AR33}, which is a null mutation in *deltaD* (Holley et al., 2000); and mib^{ta52b}, which inactivates an E3 ubiquitin ligase activity that acts on Delta proteins and is required for their ability to activate Notch (Itoh et al., 2003; Jiang et al., 1996; Schier et al., 1996). Although aei^{AR33} mutants are homozygous viable, mib^{ta52b} homozygotes are already moribund by 5 days of development. We therefore limit ourselves here to an analysis of the gut phenotype at early stages, before feeding begins at 5 days of development, but at a time when the lumen is already open, differentiation has begun, and variability resulting from differences in feeding behaviour is avoided.

DeltaC and DeltaD are both normally detected in a small proportion of gut epithelial cells in wild-type fish at 3 days – no more than two or three cells per section (Fig. 5C; and DeltaC, data not shown). We have concentrated on DeltaD, as this is the component disrupted in *aei*. Double labelling in the wild type shows that the cells positive for DeltaD are a subset of those stained with 2F11 antibody (Fig. 4C), implying that, as in the mouse, Delta expression is characteristic of secretory cells. Many 2F11-positive cells, however, do not show any DeltaD staining. This may be simply because Delta expression in these differentiating cells in the gut is transient, as it is in other tissues such as the CNS and the inner ear (Haddon et al., 1998a; Haddon et al., 1998b). Because extremely low levels of Delta protein at the cell surface are sufficient for function (Itoh et al., 2003), it may also be that some cells expressing DeltaD at functionally significant levels go undetected.

Failure of Delta-Notch signalling diverts cells towards a secretory fate

In aei^{AR33} homozygotes, as expected, no DeltaD protein was detectable (data not shown). In sections of 5-day-old larvae, the structure of the epithelium appeared normal, except for a mild excess of 2F11-stained (i.e. secretory) cells (Fig. 4D,E). To quantify this, we made counts at different levels along the gut in each of 12 wild-type and 12 aei^{AR33} larvae. As shown in Fig. 4F,G, the fraction of cells positive for 2F11 was increased in the mutant by 28-41%, depending on the region. This strongly suggests that, as a result of the loss of DeltaD, either an increased proportion of cells have become committed to the secretory fate, or there has been an increase of cell proliferation in the secretory lineage subsequent to this cell-fate choice.

To distinguish between these possibilities and to assess the effects of a more complete loss of Delta-Notch signalling, we examined the phenotype of mib^{ta52b} homozygotes. After 2 days of development (48 hours), the intestine of these mutants still appeared indistinguishable from the wild type, and no cells were stained with 2F11 antibody (data not shown). But by 3 days (72 hours), almost all (80-100%) of the cells in the mutant epithelium were 2F11 positive, when compared with 5-10% in

the wild type (Fig. 5A,B). This premature and excessive production of presumptive secretory cells was accompanied by a striking increase in expression of DeltaD, which was detected in vastly more cells than in the wild type and more intensely in the individual cells, which displayed the protein on their surfaces instead of containing it only in intracellular granules (Fig. 5C,D). This is as expected for a *mib* mutant, where Delta internalization and Notch signalling both fail (Itoh et al., 2003). We infer that the limited number of cells expressing DeltaD and 2F11 in the wild type is controlled by lateral inhibition via Delta-Notch signalling. Corresponding to the increase of secretory cells in the mutant, we found that expression of the enterocyte marker 4E8 was dramatically reduced (Fig. 5E,F); thus the increase of secretory cells was at the expense of absorptive cells. Moreover, in many sections, especially at posterior levels, the architecture of the gut epithelium was abnormal: it was several cells thick rather than simple cuboidal; 2H9 staining, normally concentrated at the basal ends of the cells, was seen apically instead (Fig. 5G,H); and the staining of the basal lamina with 5F11 antibody was reduced (Fig. 5I,J). TUNEL labelling showed that the abnormal differentiation observed in *mib* embryos at 3 days was associated with increased cell death a day later (Fig. 5K,L). Whereas no TUNEL-labelled cells were seen in the gut epithelium at either 3 or 4 days in the wild-type, and few or none were seen at 3 days in *mib*, they had become very plentiful by 4 days in the *mib* mutant. Apart from this difference in the numbers of dying cells, the 4-day-old mutants showed a phenotype similar to that of the 3-day-old mutants.

Discussion

Most previous studies aimed at understanding the control of gut cell renewal have been done in mammals. In this paper, we propose the zebrafish as an alternative model organism to address unanswered questions about this process. For this purpose, we have generated a panel of monoclonal antibodies as markers and have used them to show that the Notch signalling pathway mediates lateral inhibition to control the choice of differentiated fate in the intestinal epithelium. Although previous studies in the mouse have also implied that Notch signalling is important in the gut, our results go beyond this, and indicate that the default behaviour of intestinal epithelial cells in the absence of Notch signalling is to differentiate along a secretory pathway.

Zebrafish renew their gut lining in essentially the same way as mammals

Our BrdU pulse-chase experiments show that the pattern of cell replacement in the juvenile zebrafish gut is similar to that seen in mammals. Our findings on this point match those obtained concurrently, in an independent study, by Wallace et al. (Wallace et al., 2005). The gut lining is corrugated, with villi that protrude into the lumen and are covered with postmitotic differentiated cells. These are continually and rapidly replaced by proliferation of cells in the intervillus pockets. The pockets, which are analogous to crypts of Lieberkühn, must contain stem cells to maintain this flux, accompanied, presumably, by their dividing offspring – committed progenitors undergoing transit amplifying divisions prior to terminal differentiation. We can recognise three main classes of differentiated cells by

electron microscopy: absorptive cells (enterocytes), with a brush border; goblet cells, containing mucus; and enteroendocrine cells, which are characterized by small secretory granules. All these cell types are found in the different regions of the intestine, but in varying proportions. Paneth cells do not appear to be present [in agreement with Pack et al. (Pack et al., 1996)]. Our findings are in general agreement with those of Wallace et al. (Wallace et al., 2005), in their independent study of intestinal growth and differentiation in zebrafish, except that they have reported in addition a fourth intestinal cell type, which they propose as the analog of mammalian M cells.

At early stages, before about 5 days of development, villi have not yet formed and proliferating cells are distributed throughout the epithelium. Already at 3 days, however, some cells express molecular markers, indicating that they have begun to differentiate, as we have been able to show with our panel of monoclonal antibodies.

Monoclonal antibodies provide early markers to distinguish cell types and cell polarity in the zebrafish gut

To generate our monoclonal antibodies, we used a 'shotgun' approach, based on immunization of mice with homogenates of zebrafish gut. The relatively large evolutionary divergence (450 million years) between zebrafish and mouse presumably gives better immunogenicity than would be obtained with injections of mammalian material. Although the shotgun approach leaves us uncertain as to the molecular nature of the antigens that are recognized, it has the virtue of yielding many useful antibodies rather easily, including, in our case, markers of cell polarity as well as cell type. Particularly useful for our purposes were the three 'pan-secretory' antibodies, including 2F11, that labelled all secretory cells, both goblet and enteroendocrine. With these antibodies, we were able to reveal the pattern of gut cell differentiation as early as 3 days of development, before differentiation was apparent morphologically; thus, we could analyse the effects of the *mib* mutation before the mutant embryos died.

Distinct signalling pathways regulate stem-cell maintenance and choice of final differentiated state

Renewal of the gut epithelium involves two main types of cell-fate decision. First of all, each daughter of a stem cell must choose between remaining as a stem cell and becoming a progenitor committed to terminal differentiation. Second, if the cell becomes a progenitor, it must sooner or later choose between several alternative modes of differentiation.

Studies in the mouse indicate that the size and location of the stem cell population are controlled by the Wnt and Bmp4 signalling pathways (Haramis et al., 2004; He et al., 2004; Sancho et al., 2004). In both cases, the signal molecules come, at least in part, from the mesenchyme and act on the epithelial cells (Lickert et al., 2001). Activation of the Wnt pathway is required for development of the crypts of Lieberkühn and favours maintenance and proliferation of stem cells, while Bmp4 acts antagonistically to repress the formation of ectopic crypts along the intestinal villi.

In contrast to these signals controlling stem cell maintenance, Delta-Notch signalling in the gut occurs between one epithelial cell and another, and controls the choice of

differentiation pathway. In the mouse, *Dll1* is expressed in the cells with a secretory fate, while *Notch1* is expressed diffusely in the crypt epithelium (Schroder and Gossler, 2002), suggesting that this is where the critical signals are exchanged. Similarly, in the zebrafish, we have seen that the Delta1 orthologue DeltaD is expressed in the secretory (2F11-positive) lineage, and we have demonstrated an increase in the proportion of 2F11-positive cells in the *aei* mutant, where DeltaD is lacking. The most plausible interpretation is that Delta-Notch signalling, in this system as in many others, mediates lateral inhibition: through Delta and Notch, the cells of the secretory lineage inhibit their neighbours from becoming secretory.

A secretory mode of differentiation is the default in the absence of Delta-Notch signalling

The interpretation in terms of lateral inhibition is greatly strengthened by our observations on the *mib* mutant. Here, all Delta-Notch signalling is thought to be blocked, and a much more extreme effect is seen: almost all the epithelial cells become positive for 2F11, implying that they have adopted a secretory, instead of an absorptive, character. The weaker phenotype of the *aei* mutant is presumably a reflection of redundancy among the Notch ligands.

The *mib* mutant exemplifies very strikingly the parallels between the role of Delta-Notch signalling in the intestine and its role in neural and sensory epithelia. In each of these tissues, the mutant shows a loss of lateral inhibition, leading to a huge excess of one class of cells – secretory cells in the gut, neurons in the CNS (Haddon et al., 1998b; Jiang et al., 1996; Schier et al., 1996), hair cells in the ear (Haddon et al., 1998a; Haddon et al., 1999) – at the expense of other cell types. In each case, moreover, the failure of Delta-Notch signalling leads to an upregulation of Delta expression, implying that expression of Delta itself is normally regulated negatively by Notch activity: if a cell in the wild-type organism expresses Delta, thereby activating Notch in neighbouring cells, it will not only inhibit these neighbours from choosing the primary fate, but will also drive down their expression of Delta. This gives rise to a feedback loop that tends to amplify differences between adjacent cells so as to create a mixture of different cell types (Lewis, 1998).

It is worth noting that the intestinal epithelium of *mib* embryos was not only abnormal in its ratio of cell types, but also often appeared grossly disorganised, sometimes multilayered, with a loss of normal polarity of the epithelial cells as evidenced by labelling with the marker 2H9. These features also have parallels in other tissues (Haddon et al., 1999) and support the suggestion that Notch signalling is important for epithelial architecture as well as cell fate choices (Hartenstein et al., 1992). Perhaps the secretory cells, which are normally dispersed among non-secretory cells, lack the homophilic adhesion proteins that are needed for normal epithelial cohesion.

Our *mib* observations strongly suggest that in the absence of Notch signalling all or almost all the gut epithelial cells follow the secretory pathway of differentiation as a default. Important questions remain, however. We cannot be sure, for example, that the role of Delta-Notch signalling at 3 days of development is the same as its role in the mature gut. It is not yet clear whether intestinal stem cells are retained in *mib* mutant fish,

or whether they too depend on Notch signalling for their maintenance, like the neuroepithelial progenitors in the chick retina (Henrique et al., 1997). It remains to be seen how Notch signalling is related to the control of cell proliferation and the role of the Wnt signalling pathway. These problems are entwined with the issue of precisely where and when in the lineage of a gut cell the key cell-fate decisions are taken. Studies of normal, mutant and transgenic zebrafish, using the antibodies we have generated, should provide a route towards answering some of these basic questions about the mechanisms controlling renewal of the gut epithelium in vertebrates.

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