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Hedgehog signalling is required for cloacal development in the zebrafish embryo

CAROLINE A. PARKIN[1,2], CLAIRE E. ALLEN[1] and PHILIP W. INGHAM*[,1,2]

[1]*MRC Centre for Developmental & Biomedical Genetics, University of Sheffield, Firth Court, Western Bank, Sheffield, U.K. and* [2]*Institute of Molecular and Cell Biology, Proteos, Singapore*

ABSTRACT The Hedgehog (Hh) family of signalling molecules is essential for a wide range of developmental processes. Mammalian studies have implicated the Hedgehog pathway in the aetiology of anorectal malformations (ARMs), relatively common congenital anomalies caused by failures in the development of the cloaca. In this study we demonstrate that Hh signalling is absolutely required for the formation of the zebrafish cloaca and that the severity of the posterior gut abnormalities induced by a reduction in Hh activity is dependent on the levels of Hh signal transduction. The complete loss of all Hh activity results in the most severe defects and the critical period for Hh activity is between 34 and 74 hours post fertilisation. Using a range of mutant genotypes that cause notochord and floorplate abnormalities, we show that the source of the Hh signals required for posterior gut formation is the endoderm and not the notochord, as previously postulated in mammalian models of ARMs. We show that Adriamycin, a drug known to cause ARMs in rat, but not chick embryos, has no effect on the development of the zebrafish gastrointestinal tract. These studies establish the zebrafish as a model for ARMs, and for the elucidation of other pathways involved in hindgut developmental processes.

KEY WORDS: *zebrafish, sonic hedgehog, gut, anorectal malformations, cloaca, stenosis, Adriamycin*

Introduction

The mechanisms involved in the development of the vertebrate distal hindgut are yet to be fully elucidated. The majority of studies to date have focused on the morphology of the hindgut in mouse and human and observations of gene expression in the hindgut have, for the most part, not been accompanied by analyses of the corresponding gene function in hindgut development. Some studies in mammalian models have, however, demonstrated a role for *Sonic Hedgehog (Shh)* and *Fibroblast growth factor 10 (Fgf10).* Mice mutant for either of these genes exhibit hindgut malformations similar to those observed in human pathologies. Additionally, Hh mutants display hyperplasia of the bladder and genitals (Fairbanks *et al.*, 2004, Haraguchi *et al.*, 2000, Kimmel *et al.*, 2000, Mo *et al.*, 2001, Sasaki *et al.*, 2004b).

Anorectal malformations (ARMs) in humans encompass a variety of defects of the rectum, urinary and reproductive tracts with varying degrees of complexity and severity. The commonest and least severe defect is anal stenosis – the narrowing of the anal opening. More severe is the imperforate anus, which has an incidence of around 1 in 5000 (Mo *et al.*, 2001). Those born with imperforate anus have no anal opening at all, and instead a fistula (channel) may form between the rectum and an adjacent structure. Alternatively the rectum may end as a blind pouch, which is called atresia. The most severe and rare malformation in the spectrum, occurring in 1 in 50,000 births (Mo *et al.*, 2001), is the persistent cloaca, where a single perineal orifice is formed due to the confluence of the rectum, vagina and urethra into a common channel.

Individuals with ARMs often have abnormalities in other organ systems. Currently the pathogenesis and aetiology of these defects and the association between them is poorly understood. Malformation of the anus is associated with several congenital syndromes, including Townes-Brocks, Pallister-Hall, and Currarino syndromes, and the VACTERL (Vertebral, Anal, Cardiac, Tracheoesophageal, Renal and Limb) associa-

Abbreviations used in this paper: ARM, anorectal malformation; Hh, hedgehog; hpf, hours post fertilization; Shh, sonic hedgehog; VACTERL, vertebral, anal, cardiac, tracheosophageal, renal and limb.

***Address correspondence to:** Phillip W. Ingham. MRC Centre for Developmental & Biomedical Genetics, University of Sheffield, Firth Court, Western Bank, Sheffield, S102TN, U.K. Fax: +44-114-276-5413. e-mail: p.w.ingham@sheffield.ac.uk

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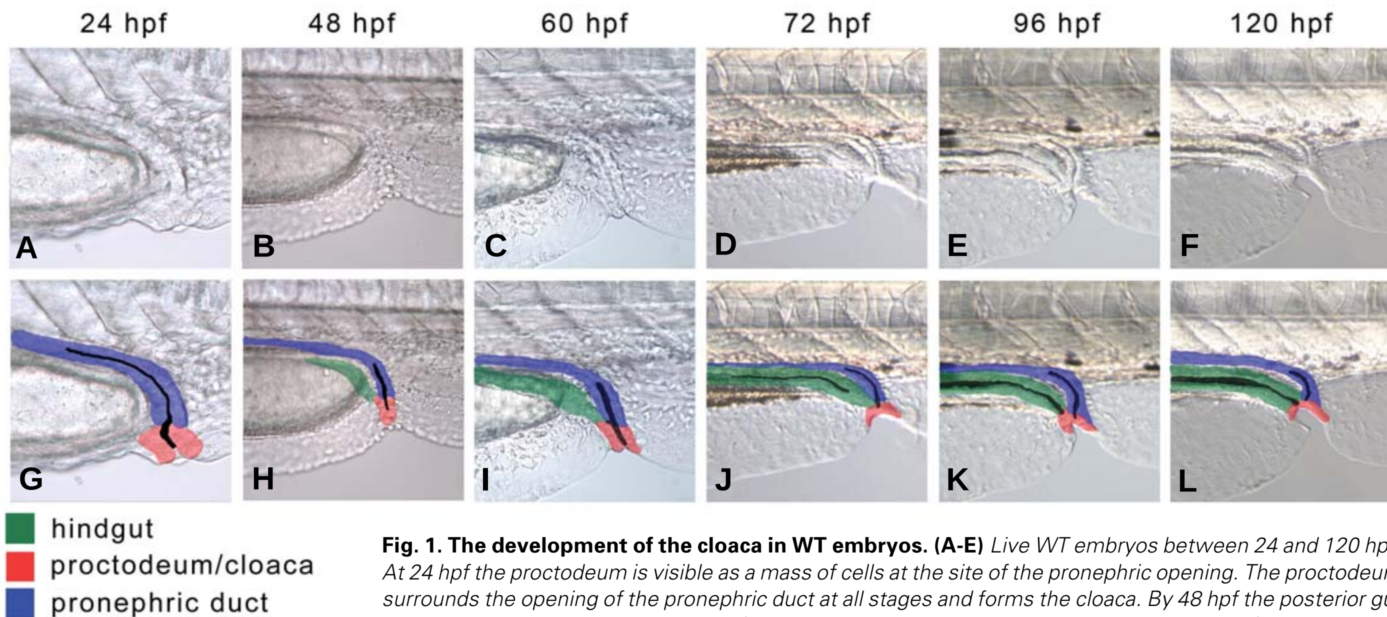


Fig. 1. The development of the cloaca in WT embryos. (A-E) *Live WT embryos between 24 and 120 hpf. At 24 hpf the proctodeum is visible as a mass of cells at the site of the pronephric opening. The proctodeum surrounds the opening of the pronephric duct at all stages and forms the cloaca. By 48 hpf the posterior gut has reached the cloaca and starts to fuse with it. As the posterior gut becomes canalised from anterior to posterior, the proctodeum invaginates to form the anus. The anal canal, the most distal part of the gastrointestinal tract, is formed by the fusion of the proctodeum with the posterior gut endoderm. By ~96 hpf the posterior gut lumen is in contact with the ventral edge of the embryo, but is not yet open. At 120 hpf the gastrointestinal tract opens externally, adjacent to the pronephric duct.* **(G-L)** *Live WT embryos between 24 and 120 hpf with an overlay showing the identity of various tissues: green, posterior gut; blue, pronephric ducts; red, proctodeum/cloaca.*

tion (Belloni *et al.*, 2000, Hagan *et al.*, 2000, Hall *et al.*, 1980, Kohlhase *et al.*, 1998, Martinez-Frias *et al.*, 2001, Rittler *et al.*, 1996, Ross *et al.*, 1998).

Animal models for various digestive system malformations have been developed to investigate their genetic and environmental basis. Previous studies in mammals have shown that *Shh* and its downstream mediators play an important role in early hindgut specification (Bitgood and McMahon, 1995, Wells and Melton, 1999). *Shh* and *Gli* deficient mice display various VACTERL-like abnormalities, including hindgut malformations such as imperforate anus and anal stenosis (Kim *et al.*, 2001, Kimmel *et al.*, 2000, Mo *et al.*, Ramalho-Santos *et al.*, 2000).

Prenatal exposure of foetal rats to the drug Adriamycin induces various malformations similar to those seen in the VACTERL association, providing a model that has been used extensively to investigate the various anomalies including the consistently occurring ARMs (Gillick *et al.*, 2003, Kolker *et al.*, 2000, Millar *et al.*, 2001, Mortell *et al.*, 2004). Several studies have shown that the expression of *Shh* is altered in the Adriamycin treated rats (Arsic *et al.*, 2004, Arsic *et al.*, 2003, Spilde *et al.*, 2003). In the foregut *Shh* expression is reduced and eventually lost when rats are treated with Adriamycin, whilst expression of *Shh* in the notochord is maintained (Arsic *et al.*, 2004). The notochord is, however, abnormal in these rats and may remain attached to the gut, or may bifurcate producing ectopic notochord. Interestingly, the increased volume of notochord per embryo means there is a relative increase in *Shh* expression compared to control rats (Arsic *et al.*, 2004). It has been speculated that the altered notochord in the treated rats is responsible for some of the VACTERL anomalies, including the ARMs, either due to changes in *Shh* expression or unknown changes in factors normally secreted from the notochord (Gillick *et al.*, 2003, Qi *et al.*, 2003). In contrast to the consistent ARMs seen in foetal rats exposed to Adriamycin, no anal defects were detectable in similarly treated chick embryos (Mortell *et al.*, 2003).

Recently Pyati *et al.* (2006) presented the first detailed description of zebrafish cloacal development and demonstrated an early requirement for Bmp signalling in specification of the presumptive cloaca. Fish deficient in Bmp activity have defects in the opening of the pronephric ducts (kidney terminus) and the posterior gut, which was attributed to an overall failure in the external opening of the cloaca. The role of other genetic pathways in zebrafish cloacal formation has not been studied.

Here we analyse cloacal development in zebrafish embryos deficient in Hh activity. The zebrafish offers unique advantages over amniote models of vertebrate development due to its external fertilisation and optically clear embryos, allowing the visualisation of gastrointestinal development at all stages in living animals.

To examine the role of the Hh pathway in posterior gut development we used mutants in *smoothened (smo)*, *shha, ihha* and *gli2a* as well as various mutants affecting notochord development that have either increased or decreased levels of midline Hh activity. To determine the temporal requirement for Hh activity we used the Hh pathway antagonist cyclopamine. The posterior guts of embryos with defective notochords were largely unaffected. Global reduction in Hh pathway activity, however, resulted in a spectrum of ARMs similar to that seen in humans, with the severity of the abnormality correlating with the residual level activity of the pathway, the most severe defects occurring in embryos devoid of all activity.

Results

The earliest arising part of the zebrafish cloaca is the proctodeum, which eventually becomes the caudal most part of the gastrointestinal tract (i.e. the anus); it also encircles the adjacent pronephric opening (Fig. 1). The proctodeum initially has a very narrow orifice, but between 60 and 96 hpf this opens out to form an inverted U-shape that surrounds a gap in the ventral fin (which must develop to create an opening for the urorectal structures) (Fig. 1C-E). The pronephric ducts open externally at the position of the proctodeum around 24 hpf (Kimmel *et al.*, 1995). At this stage, the posterior gut is a mass of endodermal cells, slightly anterior to the pronephric ducts. During the following 3-4 days of development, the endoderm arranges itself into a rod (Field *et al.*, 2003) (Fig. 1B-F). A lumen develops, advancing posteriorly from the midgut, until 96 hpf, at which point the lumen has reached the end of the posterior gut and is only closed by the proctodeum/anus (Fig. 1E). Opening of the gastrointestinal tract is not completely synchronised between embryos in the same clutch. It occurs at some point between the 4th and 5th day of development, when the controlled excretion of faeces (or injected fluorescent dye) is visible in live embryos (data not shown).

Hh pathway genes are expressed in the developing zebrafish posterior gut

The zebrafish has multiple Hh homologs (Ekker *et al.*, 1995, Krauss *et al.*, 1993), including three that are expressed in the gut endoderm. The expression of *shha* and *shhb* in the zebrafish foregut and midline tissues has been described previously (Roy *et al.*, 2001, Strahle *et al.*, 1996), however, these studies did not address expression of Hh pathway components in the posterior gut and surrounding tissues. Expression of *shha,* but not *shhb,* is detected in the developing posterior gut and proctodeum from around 24hpf (Fig. 2A) (*shhb* data not shown). Expression of *shha* in the posterior tip of the gut persists until at least 120 hpf, with transcripts becoming restricted to scattered cells in the endodermal layer (Fig. 2B, C). A third Hh gene, *ihha*, is first detectable at 12-14 somites in the developing proctodeum (Qiao, 1997; data not shown). This expression continues throughout development, with the signal being weakly maintained in the putative anal region at 24 hpf, and more strongly from 48 hpf onwards in the posterior gut and cloaca (Fig. 2D-F). Expression of the genes encoding the Hh receptor *(ptc1)*, and transcriptional effector *(gli2a)* is detected in the surrounding mesenchyme (Fig. 2G-L).

Shha *and* **Smo** *mutant fish have cloacal abnormalities*

To assess the role of Hh signalling in cloacal development, we analysed the phenotypes of fish mutant for *smoothened (smu^{h641}), shha (syu^{t4}, syu^{bx392}, syu^{bq70})* or *ihha^{hu3121}* (Barresi *et al.*, 2000, Schauerte *et al.*, 1998). Live DIC imaging allows good visualisation of zebrafish cloacal morphology but further structural details are revealed with confocal scans of actin (FITC-Phalloidin) and nuclear staining (propidium iodide; henceforth called A-PI staining). To investigate the functionality of the digestive system embryos were injected into the pericardial sac with a fluorescein salt that accumulates in the embryonic gut and is visibly excreted once the gastrointestinal tract is functional (as per comm. Leila Abbas). In cases of imperforate anus, the dye fails to be excreted and remains within the gut.

The *smu* mutant exhibits the most severe anal phenotype of all the Hh pathway mutants studied. The pronephric ducts appear to be positioned correctly at 120 hpf, although they are slightly disorganised (compare WT in Fig. 3A-C to *smu* in Fig. 3E-G). The proctodeum invaginates to form the anal canal, but it fails to open out into a characteristic inverted U-shape. Additionally, the posterior gut itself is poorly developed. Examination with A-PI staining confirms that *smu* embryos have a very narrow posterior gut, with little musculature evident (Fig. 3G). The A-PI staining also revealed that the posterior gut never fuses with the proctodeum, and instead ends blindly (atresia) in all embryos examined (Fig. 3G, H). Fluorescent dye injection supports this observation, as all the embryos examined were imperforate (100%, 40/40; Fig. 3H). It should be noted that peristalsis was not always detected in living *smu* embryos. It is

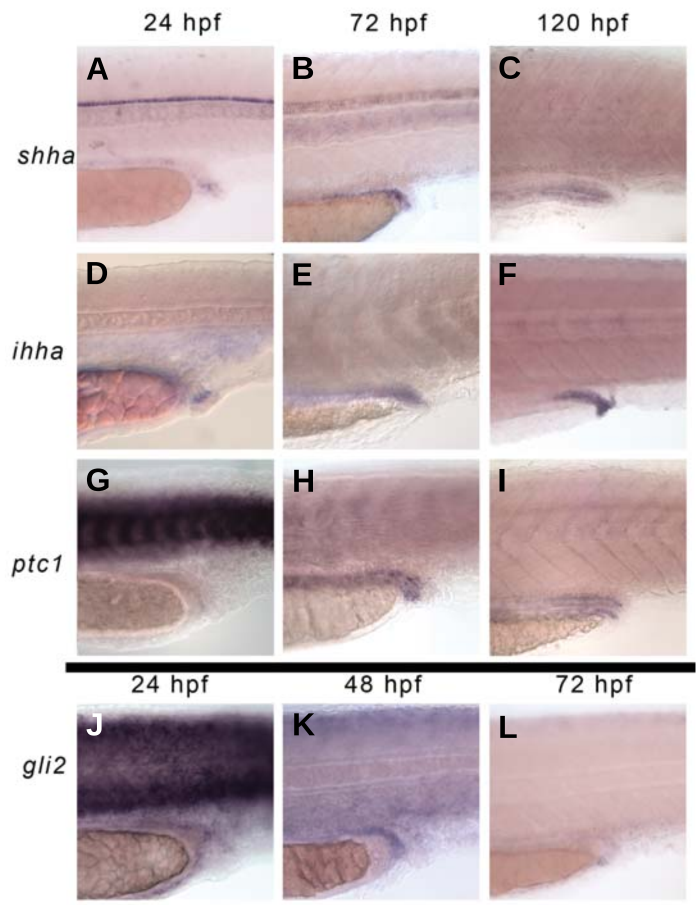


Fig. 2. Expression pattern of *Hh* genes in the developing cloaca. In situ *hybridisation stains in WT embryos.* **(A-C)** *Transcripts for* shha *are detected at low levels in the posterior gut endoderm and persist until at least 120 hpf.* **(D-F)** ihha *is expressed in the proctodeum from 24 hpf and is expressed in the posterior gastrointestinal tract and proctodeum until at least 120 hpf.* **(G-H)** *Before 48 hpf* ptc1 *transcripts are not up-regulated in the area surrounding the posterior gut, but later* ptc1 *is expressed at higher levels in the mesodermal layer that surrounds the* Hh *expressing endoderm.* **(J-L)** *At 24 hpf* gli2a *is expressed in a diffuse pattern around the developing posterior gut, around 48 hpf* gli2a *expression in the developing posterior gut is at its strongest, with low-level expression on the posterior edge of the pronephric ducts, but not within them. By 72 hpf* gli2a *is expressed in cells surrounding the anal sphincter, and weakly in a large diffuse area around the urorectal opening.* gli2a *expression is not detectable in the urorectal region after 96 hpf.*

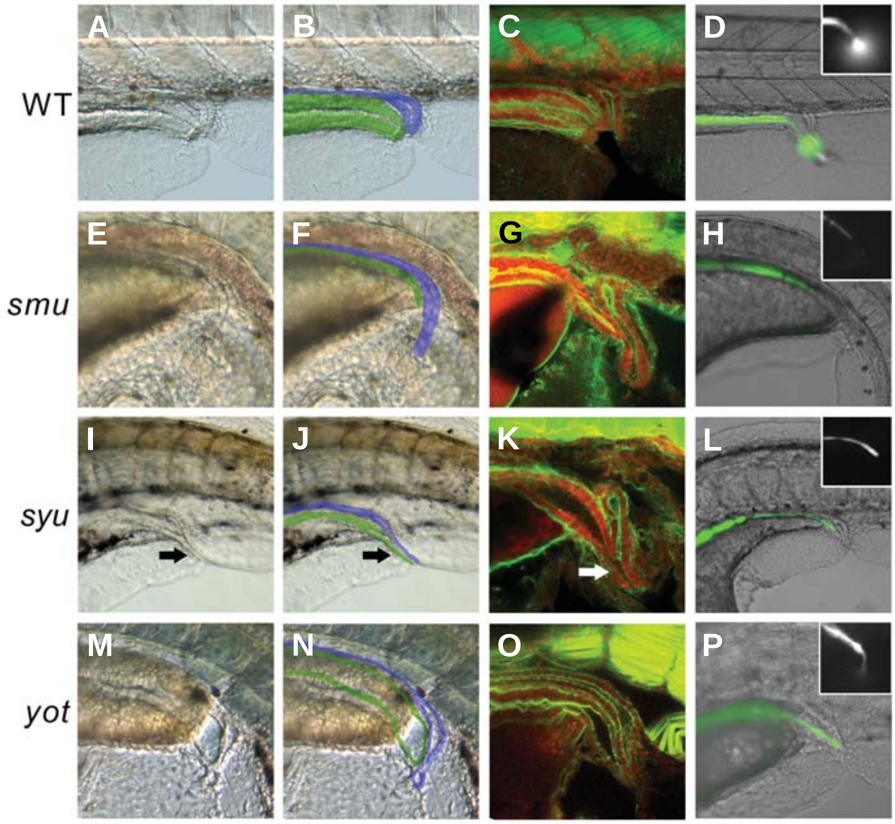


Fig. 3. Hh signalling is essential for cloacal development. *Bright field images of live Hh pathway mutants and WT siblings* **(A,B,E,F,I,J,M,N)** *and confocal scans of embryos stained with α-actin-phalloidin (green) and propidium iodide (red) at 120 hpf* **(C,G,K,O)**. *Live embryos were injected with fluorescein salt at 72-96 hpf, which accumulates in the gut prior to the opening of the gastrointestinal tract and is then excreted once the gut is functional. Brightfield/fluorescent image merge with the fluorescent channel only shown in the inset* **(D,H,L,P)**. *The overlay in* **(B,F,I,N)** *shows the approximate identity of the relevant tissues, with the posterior gut in green and pronephric ducts in blue.* **(A,B)** *WT. By 120 hpf the gastrointestinal tract has fused with the proctodeum and has an independent opening, adjacent to the pronephric ducts.* **(C)** *WT. The pronephric ducts have a single narrow opening slightly caudal to the posterior gut. The position where the ducts join is visible in the confocal cross section as an upturned 'U' shape, with the anterior ducts out of the plane of focus. The posterior gut is surrounded by smooth muscle, and ends with an anal sphincter that is only slightly narrower then the preceding gut.* **(D)** *WT embryo excreting fluorescent dye.* **(E,F)** smu. *The pronephric ducts are present and are likely to be functional, but the posterior gut is not visible, due to the shape of the embryo and folding of the ventral fins.* **(G)** smu. *The pronephric duct caudal to the point where the left and right ducts fuse is much longer in* smu *mutants because the proctodeum has not opened out. The posterior gut tries to fuse with the proctodeum, but fails, resulting in atresia.* **(H)** smu *embryos are completely imperforate (100%, 40/40) and lack peristaltic movement. Application of gentle pressure to the gut moves the dye to the end of the gastrointestinal tract, where it is clear that it is blocked and imperforate.* **(I,J)** syu^{t4} (shha). *A channel apparently common between the posterior gut and pronephros (arrow) is suggestive of a fistula between them, but it seems the gut does not fuse correctly with the cloaca leading to imperforate anus.* **(K)** syu^{t4}. *The posterior gut of* syu^{t4} *embryos ends blindly dorsal to the proctodeum in most casese. Frequently there is an abortive attempt to fuse with the cloaca leading to the appearance of a fistula between the posterior gut and pronephric duct (arrow). The gastrointestinal tract is completely imperforate – even in instances when there appears to be a fistula.* **(L)** syu^{t4} *embryo with imperforate anus/atresia; fluorescent dye can be seen at the blocked end of the gastrointestinal tract in all embryos (100%, 49/49).* **(M,N)** yot (gli2). *The pronephric ducts appear to be functional, but are slightly distorted. The gastrointestinal tract is imperforate having failed to fuse with the proctodeum. In this instance peristaltic movements have created a pressure build-up in the distal gastrointestinal tract, causing an outward bulge.* **(O)** yot. *The pronephric ducts join together in the correct position, but the usually short single duct appears slightly longer then normal due to the proctodeum failing to open. The posterior gut turns ventrally towards the proctodeum, but fails to fuse with it fully, instead ending blindly adjacent to the pronephric opening. Additionally there is a large reduction in gut girth, probably due to a reduction in smooth muscle in the caudal gut.* **(P)** yot. *The majority of* yot *embryos are imperforate (83%, 15/18), but in the embryos shown here, dye is excreted from the gut through a narrow opening, which is the equivalent of anal stenosis.*

likely that this would greatly contribute to the failure of the fluorescein to be excreted. In recently euthanized WT embryos, where peristalsis has stopped, it is possible to force open the gut and induce fluorescein excretion by applying gentle pressure with a blunt needle to the fore or midgut. Such 'forced' excretion is not possible in *smu* mutants, indicating that the gastrointestinal tract is completely imperforate.

The complete loss of Shha activity in the *syu* ^{t4} mutant results in anorectal malformations similar to, though less severe than, those observed in *smu* mutants. As in *smu* mutants, the pronephric ducts appear to develop normally but the proctodeum does not open into an upturned U-shape (Fig. 3I, J). The proctodeum remains closed at its ventral edge, as a consequence of which the gastrointestinal tract seems to fuse with the pronephric ducts; this results in what appears to be a fistula, visible in both live and fixed embryos (arrow in Fig. 3I-K). Injection of fluorescein into live *syu* ^{t4} embryos, reveals that the gut is imperforate, as no excretion of fluorescein is detectable (49/49; Fig. 3L). Embryos mutant for the moderate hypomorphic allele, *syu* ^{bx392}, have a phenotype similar to *syu* ^{t4}, although slightly less severe; only a third are imperforate (4/12), with the remainder exhibiting anal stenosis (8/12) (data not shown). Embryos homozygous for the weak hypomorphic allele *syu* ^{bq70} allele display a variable phenotype. Nearly half the embryos develop a functional and perforate anus (21/39), and a further third are perforate, but exhibit anal stenosis (12/39), while in the remaining 15% (6/39) of embryos the phenotype is similar to that observed in *syu* ^{t4} mutants, with complete atresia (data not shown).

Embryos homozygous for an ENU induced point mutation in *ihha* do not exhibit ARMs and are indistinguishable from their WT siblings (data not shown). Simultaneous homozygosity for this *ihha* allele and *syu* ^{t4} did not increase the severity of the *syu* ^{t4} phenotype (data not shown).

In the chick embryo, expression of *bmp4* in the hindgut is regulated by Hh signalling. To explore whether a similar relationship underlies the Hh mutant phenotypes in zebrafish, we analysed the expression of *bmp4* by *in situ* hybridisation. No changes were seen in the levels of expression in *smu* or *syu* mutants (Fig. 4E-G, I-K). By contrast, expression of *ptc1,* a known target of the Hh pathway is lost in the posterior gut of *smu* mutants (Fig. 4H), although it persists in *syu* mutants (Fig. 4L). Cloacal expression of *evx1* and *prdm1* is lost embryos with impaired BMP signalling (Pyati *et al.*, 2006) whereas in *syu* and *smu* mutants it persists, highlighting the altered shape of the cloaca in both mutants (Fig. 5).

A dominant repressor form of Gli2a interferes with anorectal development

The *you-too* ^{ty11} *(yot)* mutation results in expression of a C-terminal truncated Gli2a protein that is unable to activate transcription but instead functions as a constitutive repressor of Hh target genes (Karlstrom *et al.*, 2003). *yot* mutant embryos have severe anorectal defects. The gastrointestinal tract is imperforate in most cases, though as in *syu* and *smu* mutants, the pronephric ducts appear to be largely unaffected (Fig. 3M, N). In the embryo shown in Fig. 3M, pressure has built up in the gut due to peristaltic movements, causing the posterior gut to become distended. Examination with A-PI also shows an imperforate phenotype and highlights the thin posterior gut walls, which lack thick smooth muscle (Fig. 3O). Fluorescent dye injection demonstrates that most *yot* embryos are imperforate (15/18), while those that are perforate have anal stenosis (3/18; Fig. 3P). By contrast, embryos homozygous for the *dtr* mutation, which disrupts zebrafish *gli1,* show no anorectal defects were and have normal perforate gastrointestinal tracts (data not shown).

Hh dysfunction does not affect apoptosis in the urorectal region

TUNEL stains of WT embryos reveal specific apoptotic events in the proctodeum between 24 and 120 hpf (Fig. 6A-E), suggesting that programmed cell death is required for urorectal morphogenesis. TUNEL staining in *syu* and *smu* embryos did not reveal any dramatic changes in the levels or distribution of cell death in the posterior gut area (Fig. 6F-P). Counts of apoptotic cells performed at various time points show that the level of cell death varies considerably between fish but there was no significant difference between the mutant and WT embryos (Fig. 6P). By contrast, we observed a massive increase in cell death in the neural tube of mutant embryos, most noticeably at 24hpf (Fig. 6A compared to Fig. 6F, K) consistent with previous reports (Chen *et al.*, 2001).

Cyclopamine mediated knockdown of Hh signalling reveals temporal requirements during posterior gut development

To investigate the temporal requirements for Hh signalling during posterior gut development, we used the alkaloid cyclopamine to inhibit the activity of Smo (Fig. 7). Exposure to cyclopamine, (followed by its subsequent washing out before 24 hpf) caused U-shaped somites and ventral body curvature, a phenotype typical of fish with reduced Hh activity, but did not induce any strong ARMs. Exposure of embryos to 25μM cyclopamine from 24 to 48 hpf, resulted in some embryos with ARMs (Fig. 7A-D). The posterior gut looked disorganised in both live embryos and those stained with A-PI (Fig. 7A-C) and there appeared to be a single outlet for both the gastrointestinal tract and pronephric duct (Fig. 7C). Injection with fluorescein revealed that a quarter had imperforate anus (4/12; Fig. 7D). In embryos treated between 34 and 74 hpf a fistula frequently developed between the gastrointestinal tract and pronephric ducts, which is clearly visible at 120 hpf in live and A-PI stained embryos (Fig. 7E, F, G). A large number of the embryos that had a single channel for the gastrointestinal tract and pronephric duct were imperforate. Fluorescein was pushed into the pronephric ducts, an effect never observed in WT embryos or those treated between 24 and 48 hpf (compare Figs. 3D, 7D to Fig.

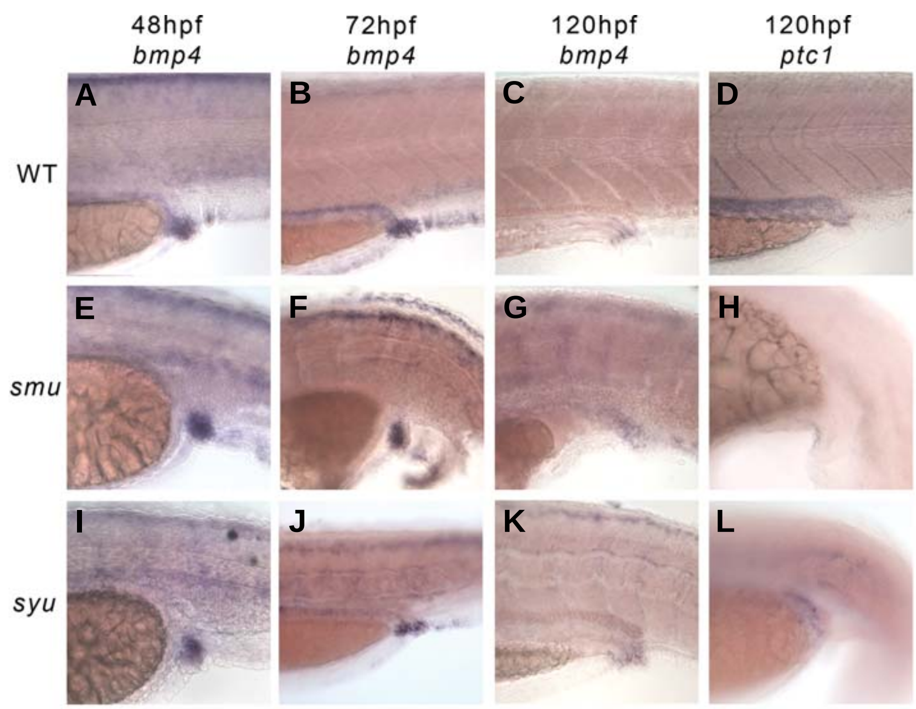


Fig. 4. Expression of putative *Hh* target genes in Hh mutants. Bmp4 *and* ptc1 *expression in Hh pathway mutants and WT siblings.* **(A-C)** *WT siblings, 48, 72 and 120 hpf.* bmp4 *is diffusely expressed in the proctodeum and posterior gut. By 120 hpf expression is restricted to the anus.* **(D)** ptc1 *expression in mesenchyme surrounding the posterior gut in a WT sibling.* **(E-G)** smu, *48, 72, 120 hpf. Although the morphological defects in the* smu *posterior gut alter the shape of the* bmp4 *expression domain, there are no changes in the levels expressed.* **(H)** *There is no* ptc1 *expression around the cloacal region with the loss of all Hh signal transduction in the* smu *mutant.* **(I-K)** syu, *48, 72, 120 hpf. Expression of* bmp4 *in the* syu *mutant is little different to the WT siblings.* **(L)** *Some* ptc1 *expression surrounding the posterior gut remains after the loss of* shha *in* syu *mutants.*

7H). Just over half the embryos in this treatment window were perforate (10/18) and over half of these embryos (6/10) displayed a fistula (6/18 of total embryos). The remaining 44% (8/18) of embryos had an imperforate anus and although half of these embryos had a fistula to the pronephric duct (4/8), there was no ejection of fluorescein into the surrounding water (Fig. 7H). Embryos treated between 48-74 hpf and 48-96 hpf also exhibited a large number of fistulas and atresia (Fig. 7I-L; data for 48-74hpf not shown). The fistulas occurred close to the correct anorectal opening such that there was no long, single channel composed of both posterior gut and pronephric duct, a phenotype that is frequently observed in *syu* mutant embryos and those treated with cyclopamine at earlier timepoints (Fig. 3I, K, 7A-H). In all embryos treated before 96 hpf, the proctodeum failed to adopt its distinctive upturned U-shape. A later treatment between 74 and 96 hpf resulted in no obvious ARM, although when examined with A-PI staining, compared to WT the urorectal area was somewhat disorganised (compare Fig. 3A-C with Fig. 7M-O). Fluorescein injections demonstrated that the posterior gastrointestinal tract was perforate in most cases (18/20; Fig. 6T). There was, however, some stenosis, a quarter of the embryos having a narrow anorectum (5/20).

Treatment of embryos with 5 μM cyclopamine from 24 or 48 hpf resulted in malformed proctodeums and atresia (5/15), or in two cases apparent fistulas to the pronephric ducts (2/15), similar to embryos treated with 25 μM of cyclopamine. There were no ARMs in embryos treated with 5 μM cyclopamine from 72 hpf.

Defects in the notochord and floorplate do not disrupt anorectal development

Since Hh proteins can act over many cell diameters, distantly located *Hh* expressing tissues may have an impact on posterior gut development (Gritli-Linde *et al.*, 2001). Overlying the gut during its development are the notochord and floorplate. *shha* is expressed in the notochord and medial floorplate (MFP) (Krauss *et al.*, 1993), whilst *shhb* is expressed in the MFP (Ekker *et al.*, 1995) and *ihhb* is expressed solely in the notochord (Currie and Ingham, 1996). *ihhb* and *shhb* expression in the MFP and notochord is lost by around 24 hpf.

To determine whether Hh proteins, or indeed other signalling molecules emanating from the axial midline structures of the embryo are required for the correct development of the urorectum, several mutants that disrupt the notochord and/or floorplate were studied.

In *no tail (ntl^{tc41})* mutant embryos the tail does not form and the notochord is completely missing from the posterior part of the embryo (and therefore *shha* expression is reduced to the MFP), yet despite these defects most embryos have a functional gastro-intestinal tract (11/12; Fig. 8A-D).

Zebrafish *floating head (flh)* mutants lack a notochord and are severely truncated, resembling *ntl* mutants (Talbot *et al.*, 1995). However, in addition to never developing a notochord they also have a greatly reduced floor plate. Despite the loss of nearly all midline (excluding the gut) Hh expression

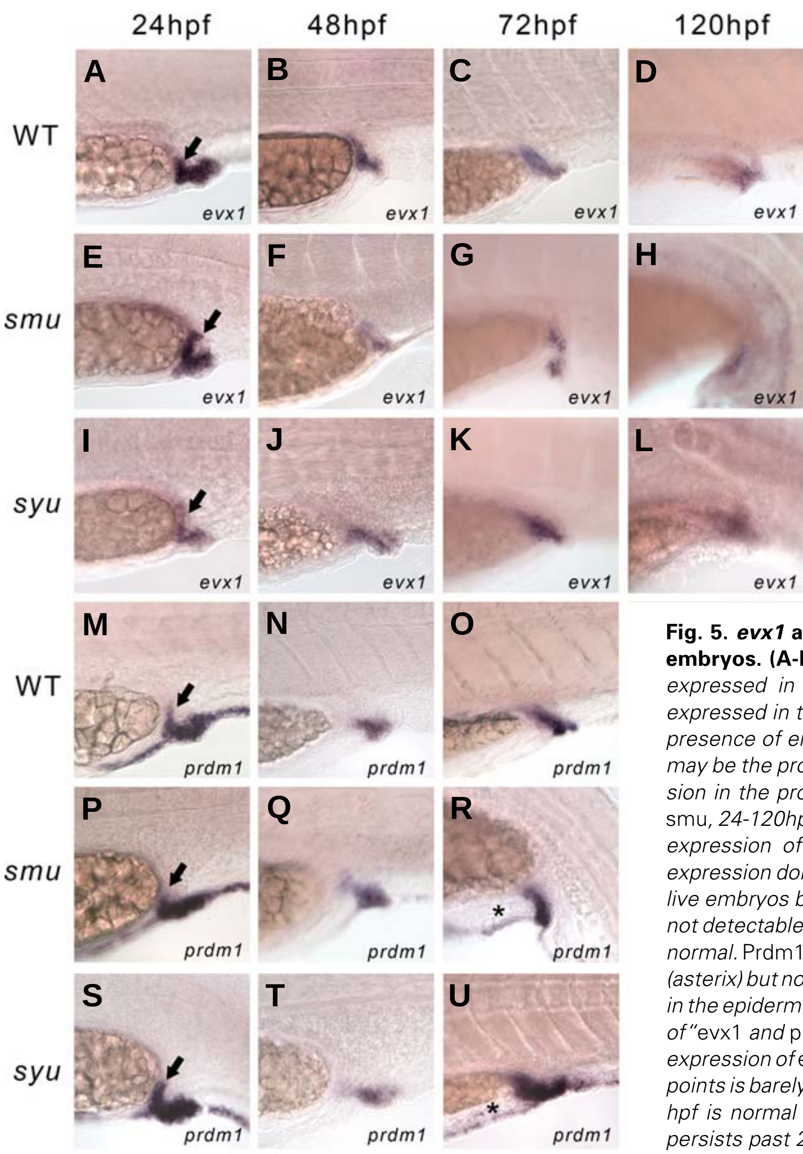


Fig. 5. *evx1* and *prdm1* expression in the cloaca of Hh pathway mutant embryos. (A-D, M-O) *WT sibling, 24-120 hpf. At 24 hpf* evx1 *and* prdm1 *are expressed in the proctodeum and caudal pronephros. They may also be expressed in the posterior gut at this stage, but it is difficult to determine the presence of endoderm in this area at 24hpf. Expression marked by the arrow may be the proctodeal tissue that fuses with the gastrointestinal tract. Expression in the proctodeum/cloaca is maintained until at least 120hpf.* **(E-F, P-R)** smu, *24-120hpf. Despite morphological changes to the proctodeum and cloaca expression of* evx1 *and* prdm1 *are maintained. The altered shape of the expression domain appears to reflect the change in shape of the cloaca seen in live embryos but not a loss of the tissue itself. At 24 hpf–*prdm1 *expression is not detectable in the putative posterior gut (arrow) whereas* evx1 *expression is normal.* Prdm1 *expression in the epidermis persists past 24 hpf in* smu *mutants (asterix) but not in WT siblings, suggesting that* prdm1 *is regulated by Hh activity in the epidermis, but not the procotdeum.* **(I-L, S-U)** syu, *24-120hpf. Expression of "*evx1 *and* prdm1 *are maintained in the cloaca of* syu *mutants. At 24hpf the expression of* evx1 *appears to be weaker than the WT siblings, but at other time points is barely distinguishable from the siblings, whilst* prdm1 *expression at 24 hpf is normal in* syu. *Like* smu *mutants* prdm1 *expression in the epidermis persists past 24 hpf (asterix).*

(Odenthal *et al.*, 1996), the *flh* gastrointesitinal tract and pronephric ducts open in the correct position (31/31; Fig. 8E-H). Fluorescent dye is extruded normally in most mutants (20/23; Fig. 8H). Although the pronephric ducts are not clearly visible in live embryos due to epidermal thickening of the ventral fins obscuring the view, examination with A-PI shows the pronephric ducts open normally and the anus is well formed (Fig. 8G).

Several other mutations affecting notochord development were investigated: *doc*, *bashful (bal)*, *sleepy* (*sly,* data not shown) and *crash test dummy (ctd)*. Embryos homozygous for all of these mutations, except *ctd*, have a short body axis, with reduced mesoderm in the tail. Nearly all the notochord and floorplate mutants studied develop functional digestive systems with perforate anus. As with WT embryos, a small number of embryos exhibited imperforate anus, but the presence of these anorectal abnormalities was not correlated with the severity of the notochord phenotype.

In the *bal* and *doc* mutant embryos, the notochord is mostly absent, although some vacuolated cells are present. This variability provides a basis for investigating the local requirement for notochord. The digestive tract, including the anorectum, is not visibly different between regions where the notochord is present and the regions from which it is absent (Fig. 8I-L).

In *ctd* mutant embryos the digestive system and urorectal opening develops normally. The notochord in *ctd* mutants undulates, which means that in some instances the notochord is closer to the digestive tract then in WT embryos. The juxtaposition of the notochord next to the digestive tract, at any anterior-posterior level, does not lead to morphological changes in the anus or the digestive tract (14/14; Fig. 8M, N).

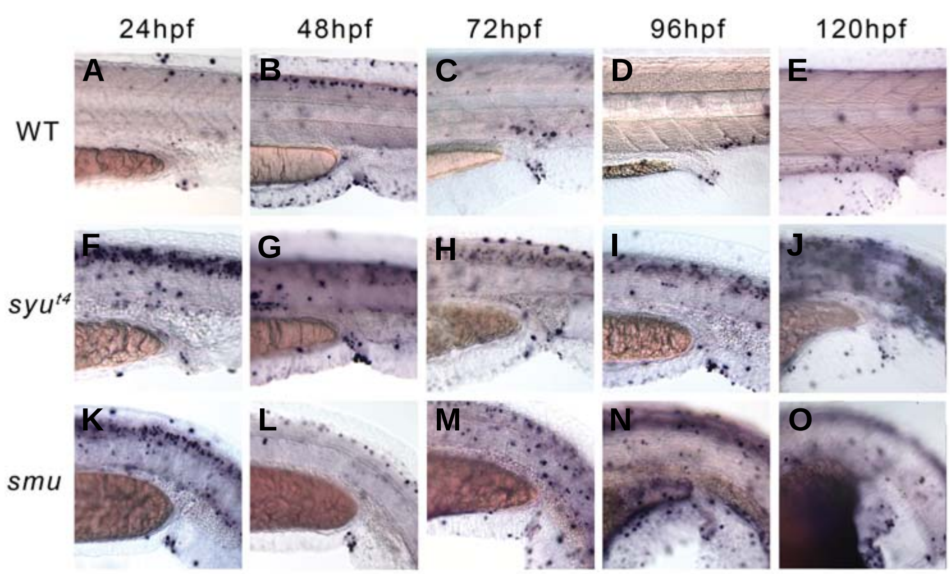
Ectopic floorplate does not affect posterior gut development but changes in mesodermal movements affect the proctodeum

Spadetail (spt) embryos have an expansion of notochord markers, including *shha* (Amacher *et al.*, 2002). The medial floorplate in *spt* embryos is also wider, leading to an overall increase in midline Hh signalling (Griffin *et al.*, 1998). The expansion of Hh expressing tissue resembles the effects seen in the Adriamycin rat model, in which the notochord is often bifurcated, producing an overall increase in Hh expression at the midline (Gillick *et al.*, 2003). As a result of the floor plate expansion in *spt* mutants, the mesoderm and its derivatives are variably affected - often the pronephros fails to form properly and may not reach the urorectal opening (6/12; Fig. 8O, P). Despite the effect on the pronephric ducts and the occasional bifurcation of the foregut, the posterior gut is always in the correct position and in most cases is functional, as evidenced by the excretion of fluorescein salt (41/42; Fig. 8R). Examination of embryos with A-PI staining revealed that, even without correctly positioned pronephric ducts, a perforate anus develops (3/3 lacking pronephric ducts; Fig. 8Q). The proctodeum in all *spt* mutants is, however, malformed, resulting in a wide gut opening (Fig. 8O, P). When pronephric ducts are present they appear at some distance from the anus, resulting in a 'spread-out' appearance to the urorectal area (Fig. 8O, P).

Adriamycin does not cause ARMs in zebrafish

Adriamycin (Doxorubicin Hydrochloride) is routinely used to induce ARMs in rats, though its mechanism of action is not well understood (Gutteridge and Halliwell, 2000). In the chick, Adriamycin has been shown to have no effect on the developing urorectum (Mortell *et al.*, 2003). In the rat, the drug is administered peritoneally to the mother, whilst the chick is exposed directly to the drug, either through an airsac or albumin injection.

As zebrafish embryos develop externally, drug treatments can be administered directly, either by injection or soaking. We used both methods to



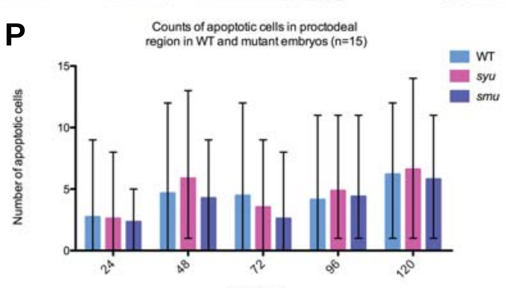


Fig. 6. Apoptotic events in the developing posterior gut of wild type and Hh pathway mutants. **(A-E)** *TUNEL staining of WT embryos between 24-120 hpf reveals the normal pattern of apoptosis during the formation of the posterior gut and pronephric ducts. At 120 hpf a number of cells in the cloaca die creating an externally opening posterior gut.* **(F-J)** *TUNEL staining of* syu *mutant between 24-120 hpf. (K-O) TUNEL staining of* smu *mutants between 24-120 hpf.* **(P)** *Counts of apoptotic cells in the mutants and WT siblings reveals that there are large differences in the amount of cell death between embryos of the same age. However, there is no significant difference between the number of apoptotic cells in WT siblings and mutant embryos.*

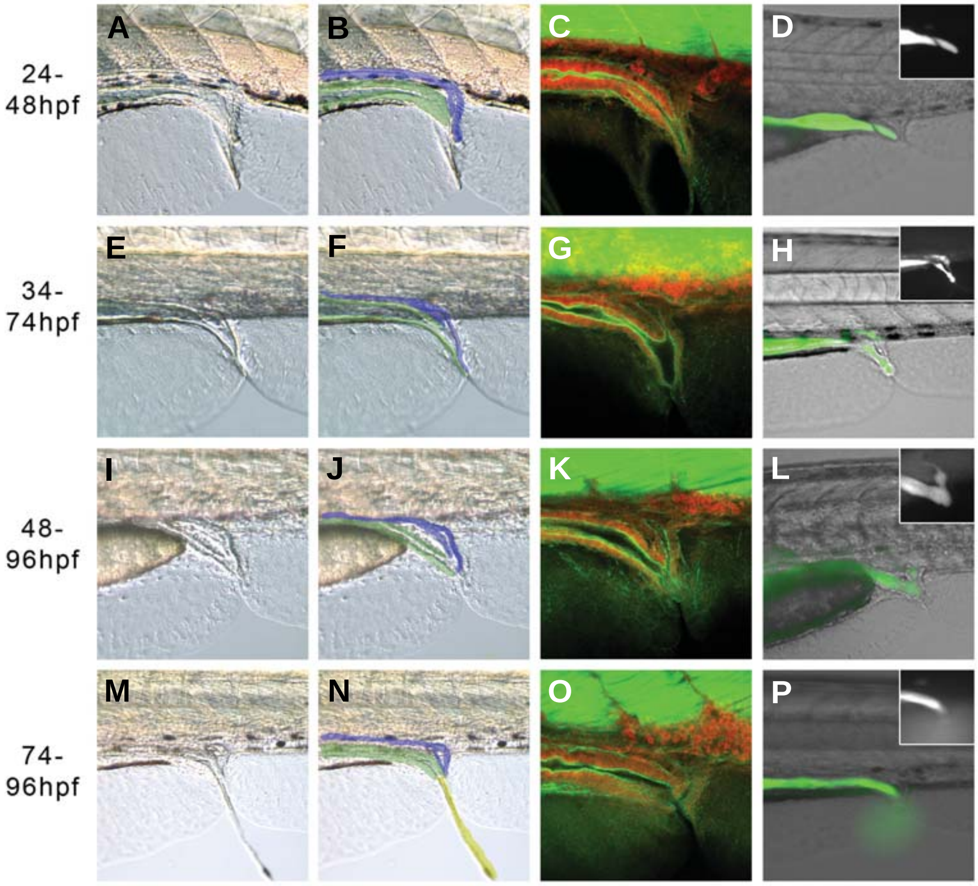


Fig. 7. Hh signalling is required between 48-74 hpf for the development of a perforate anus. *Embryos treated with 25 μM cyclopmine and imaged live at 120 hpf with bright field illumination,* **(A,B,E,F, I,J,M,N)** *or by confocal microscopy following staining with α-actin-phalloidin (green) and propidium iodide (red)* **(C,G,K,O)**. *The overlay in* **(B,F,J,N)** *shows the approximate identity of the relevant tissues, with the posterior gut in green and pronephric ducts in blue.* **(D,H,L,P)** *Merged brightfield/fluorecent images of live embryos injected with fluorescein salt at 72-96 hpf, with the fluorescent channel only shown in the inset.* **(A,B)** *After treatment from 24-48 hpf, the posterior gut is thinner than in WT embryos, and despite the appearance of a fistula* **(C)**, *the majority of embryos have a perforate gut, while 33% (4/12) were imperforate and the dye stayed in the blind ended posterior gut* **(D)**. **(E-H)** *34-74 hpf treatment. The posterior gut formed a fistula with the pronephric duct in most embryos, which allowed excretion through a single opening in the proctodeum in around half the embryos (10/18). In the embryos that were not perforate, fluorescein entered the pronephros, but could not be excreted and so moved anteriorly through the pronephric tubes, due to peristaltic pressure* **(H)**. **(I-L)** *48-74hpf treatment. Embryos from this treatment window had a range of phenotypes, 67% (16/24) were perforate, but around half had a fistula to the pronephros (14/24), which itself was not always perforate leading to fluorescein in the pronephric ducts* **(L)**. *Other embryos had atresia or stenosis.* **(M-P)** *74-96 hpf treatment. The majority of embryos are perforate (90%, 18/20 and the posterior gut musculature affords some control over the excretion of faecal matter.* **(P)** *Faecal matter being expelled in a live embryo.*

expose embryos to Adriamycin. Injections at the 1-4 cell stage did not cause any defects in the embryos (data not shown). Embryos soaked in Adriamycin from the 1-4 cell stage until 120 hpf also exhibited no malformations (data not shown). At the very highest doses of Adriamycin (300 μM) the embryos began to disintegrate at 24 hpf, presumably due to non specific toxicity. All the embryos that were soaked in Adriamycin had red intestinal lumens, indicating that the drug was being efficiently taken up. Since Adriamycin fluoresces, embryos that were either injected or soaked in the drug fluoresced brightly under UV light, indicating the drug was present in all the tissues, not just the gastrointestinal tract.

Discussion

Hh signalling is essential for anorectal development in the zebrafish

We have shown that, similar to the mouse (Mo *et al.*, 2001) zebrafish cloacal development is dependent on Hh activity. Embryos lacking all Hh pathway activity, such as those mutant for *smu* or treated with cyclopamine, display the most severe ARMs. Embryos lacking *shha* activity alone have strong malformations that resemble those found in *Shh* mutant mice (Mo *et al.*, 2001). The atresia seen in *smu* and some *syu* mutants resembles the phenotype of *Fgf10* mutant mice, whereby the mutants have a relatively normal urogenital tract but the rectum does not fuse with the proctodeum and instead ends blindly (Fairbanks *et al.*, 2004). One would expect *Smo* mutant mice to resemble closely those lacking *Shh* or *Fgf10*, however, an anorectal phenotype has not been described, presumably because *Smo* mutant embryos die very early in development (9.5 days post coitum) (Zhang *et al.*, 2001), before the gut has fully formed.

Understanding how ARMS develop requires a knowledge of how the urorectal area normally develops; however, the mechanism by which the mammalian cloaca forms the urorectal sinus and anal canal is still a contentious issue (Sasaki *et al.*, 2004a). The external development of zebrafish embryos allows the urorectal area to be examined *in vivo* as it matures. The lack of understanding of mammalian development means, however, that direct comparisons of how ARMS arise morphologically in fish and mammals is difficult, although this study demonstrates that the genetic pathways involved are conserved.

In zebrafish the presumptive cloaca or proctodeum forms from a single vacuolated cell, to which the pronephros fuses first, followed by the later developing posterior gut (Pyati *et al.*, 2006). The gut and pronephros have discrete openings into the cloaca, which starts out as an upturned U-shape and then spreads out to open up at the edge of the ventral fins. The presence of apoptotic cells in this region suggests that cell death shapes the opening of the cloaca. The ARMs arising from perturbed Hh activity appear to affect the morphological development of the cloaca rather than the regulation of apoptosis in the area, as there is no significant change in the number of apoptotic cells in the mutants.

In *syu* fish it seems that the proctodeum fails to 'spread out' in to an upturned U-shape, causing the posterior gut to become continuous with the most distal part of the pronephros. If this is the mechanism causing the ARMs in *syu* embryos then the defect cannot really be classed as a fistula as it is not a 'channel' per se, although the result is the same. the gut and pronephros share a common cavity. In *smu* mutants, the posterior gut fails to canalise completely, resulting in atresia. However, it is unclear whether this failure is within the gut endoderm, or in the proctodeal component of the cloaca, which would normally invaginate to meet and fuse with the advancing gastrointestinal lumen, but fails to do so in *smu* mutants. The proctodeum is still present in *smu* mutants (Fig. 5) and seems to sustain its rostral expansion towards the posterior gut, suggesting the fusion itself fails (either from the proctodeal or gastrointestinal side, or both). In the absence of significant changes in the levels of apoptosis and putative Hh target gene expression in the procotdeum, the mechanism causing ARMs in the Hh mutants remains unclear.

The different phenotypes observed in embryos lacking only Shha activity as apposed to all Hh activity, in the *syu* and *smu* mutants respectively, suggests that another Hh protein contributes to the development of the anorectal area. However, the loss of the only other Hh gene expressed in the posterior gut, *ihha*, does not cause ARMs, and when lost in addition to *shha* does not cause a *smu*-like phenotype (data not shown). This suggests another Hh signal is active in the area. *Shhb* is expressed in the anterior gastrointestinal tract, and it may be that low level expression extends posteriorly in *syu* mutants, or that *dhh,* which is detectable by RT-PCR, but not *in situ* hybridization is expressed in the urorectal area (Avaron *et al.*, 2006).

High levels of Hh activity are necessary for correct anorectal development but the notochord is not required to provide a source of Hh protein

Studies using Adriamycin or ENU rat models of ARMs have suggested that the notochord abnormalities induced by these teratogens underlie the anorectal defects seen in treated animals (Arsic *et al.*, 2004, Gillick *et al.*, 2003, Mortell *et al.*, 2004, Qi *et al.*, 2003). It has also been suggested that the ectopic notochord would lead to a relative increase in Shh signalling, which may be responsible for the ARMs seen in these models (Arsic *et al.*, 2004, Mortell *et al.*, 2004). Since Hh proteins can act as morphogens, eliciting differing responses in target cells as a function of their concentration (Ericson *et al.*, 1997, Ingham and

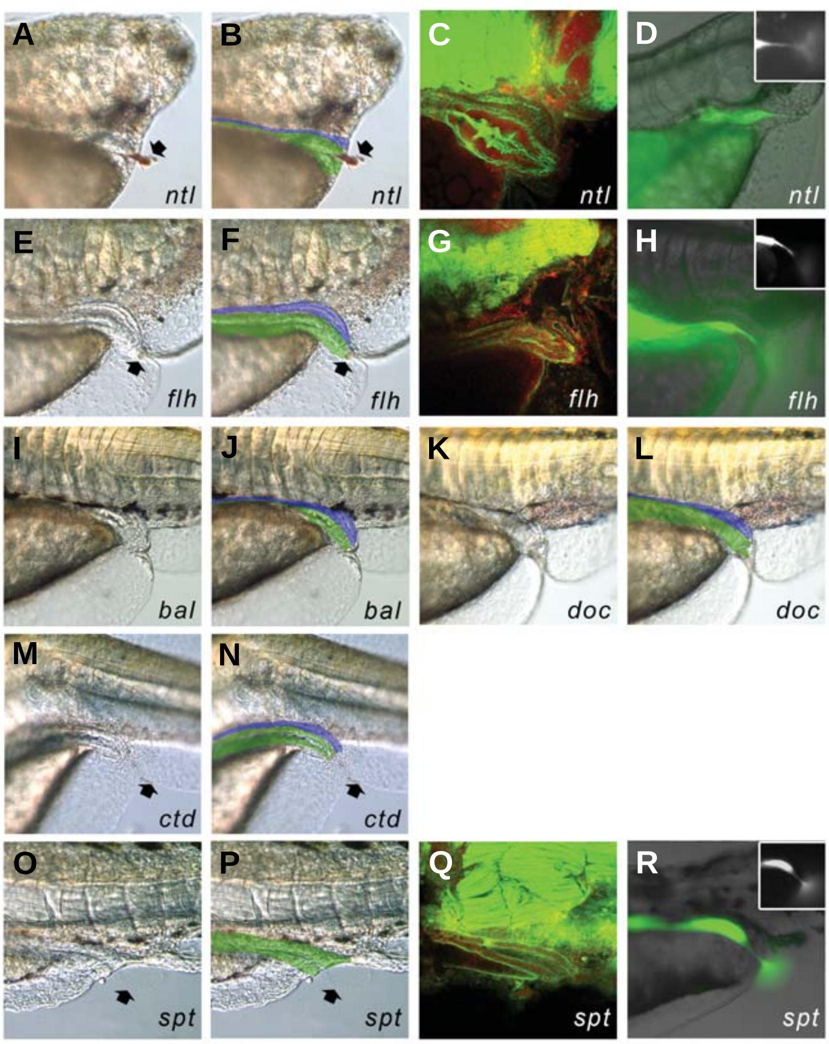


Fig. 8. Anal development in mutants with disrupted notochords. *Brightfield images of live embryos* **(A,B,E,F,H,I,L,M)** *and confocal scans of embryos stained with α-actin-phalloidin (green) and propidium iodide (red) at 120 hpf* **(C,G,Q)**. *Live embryos were injected with fluorescein salt at 72 hpf,. Brightfield/fluorecent image merge with the fluorescent channel only shown in the inset* **(D,H,R)**. *The overlay in* **(B,F,J,L,N,P)** *shows the approximate identity of the relevant tissues, with the posterior gut in green and pronephric ducts in blue.* **(A-D)** ntl *embryos have normal pronephric ducts that open adjacent to the posterior gut. The posterior gut is much thicker in both* ntl *alleles, but still opens with a normal sized orifice (91% perforate, n11/12).* **(E-H)** flh *mutant embryos develop a perforate anus and pronephric duct, which open independently of one another. (87% perforate, n20/23).* **(I-L)** *The presence or absence of a notochord near the urogenital opening does not affect its development in* bal *and* doc *embryos, which develop a functional anus.* **(M,N)** ctd *embryos develop a normal anus and pronephric opening despite undulations in the notochord close to the anus.* **(O-R)** spt *embryos have varying pronephric defects, and in some cases lack them altogether; even in the absence of the pronephros (as shown here) the development of the posterior gut is relatively unperturbed (95% perforate, 40/42), although the anal sphincter appears to be absent in most embryos* **(A,B,E,F,I,J,Q,R)** *black arrows mark the visible ejection of faecal matter from the anus.*

Fietz, 1995, Struhl *et al.*, 1997) proposals that axial midline derived Hh activity is required for anorectal development would imply that the target tissues respond to a low dose of Hhs, as the urorectal area is some distance from the notochord. Two lines of evidence presented here indicate that the notochord is dispensable for anorectal development and that high levels of Hh activity are required for normal development.

The various phenotypes of the three *syu* alleles and the *smu* mutant appear to reflect an increase in severity of ARMs with decreasing levels of Hh activity – suggesting that the levels of Hh activity are critical for posterior gut development. High levels are essential for normal development, although at lower levels some aspects of anorectal development are able to proceed. This interpretation is supported by the development of imperforate and stenotic anus in many of the embryos treated with very low levels of cyclopamine. Five μm of cyclopamine was previously shown to be sufficient to inhibit only the highest levels of Hh signalling in the somites (Wolff *et al.,* 2003). If any of the Hh signalling required for normal anorectal development originates from the midline than we would have expected to see ARMs in the mutants with reduced notochord and/or floorplate similar to those seen in low-dose cyclopamine treated embryos. The loss of Hh signals emanating from the midline should have reduced the total amount of activity in the urorectum if it were normally signalling to the gut, yet none of these mutants have significant ARMs, demonstrating that midline Hh signalling is dispensable for anorectal development.

It is also possible that the notochord is not involved in the formation of ARMs in other animals. The ARMs seen in zebrafish Hh mutants closely resemble those seen in mice and rats, and even humans. The hindgut is also a source of Hh signalling in these species and so it is very likely that some of the ARMs are induced by changes in Hh expression in the hindgut itself.

Interestingly, the increase in Hh activity occuring in the *spt* mutant did not cause anorectal defects resembling the ARMs seen in Adriamycin and ENU treated rats, in which Hh activity is postulated to be increased. Other studies investigating the nature of oesophageal and tracheal deformities in Adriamycin exposed embryos have suggested that changes in *Shh* expression in the foregut itself are responsible for these defects (Arsic *et al.*, 2004, Arsic *et al.*, 2003, Ioannides *et al.*, 2003) and oesophageal development is also perturbed in Hh deficient zebrafish embryos (Wallace and Pack, 2003). By analogy, it seems plausible changes in Hh expression in the posterior gut underlies the teratogen induced ARMs .

Zebrafish gli *mutants are comparable to those seen in amniotes*

The zebrafish *gli1* mutant, *dtr*, has a mostly WT posterior gut, with only a mild stenosis phenotype and the possible loss of the anal sphincter. *Gli1* mouse mutants do not have ARMs (Mo *et al.*, 2001), suggesting that *Gli2a* and *Gli3* are able to compensate for the loss of *Gli1*, during posterior gut development, in both mouse and fish.

The zebrafish *yot* mutation generates a dominant repressor form of Gli2a, which results in a subsequent decrease in Hh target gene expression. Consistently, *yot* embryos have strong ARMs, with anal atresia or anal stenosis. *gli2a* transcripts are detectable in the posterior gut during development, suggesting that it normally transcribes Hh target genes in this region.

In the mouse Gli2a is the major transcriptional activator. *Gli2a* mutant mice have imperforate anus, with either recto-urethral or rectal-vaginal fistula (Mo *et al.*, 2001). Both the fish and mouse mutations result in a decrease in Hh target transcription and exhibit similar phenotypes – although *yot* mutants lack the fistulas. It seems probable that *gli1* and/or *gli3* are able partly to compensate for the dominant repressor actions of the mutant *gli2a* in *yot* embryos.

Interestingly, the truncated protein generated by the *yot* mutation resembles the repressor form of Gli3 that leads to dominant Pallister-Hall syndrome in humans (Kang *et al.*, 1997). Studies have shown that only a mutation in the middle region of the gene causes Pallister-Hall syndrome, mutations elsewhere generally cause Greig cephalopolysyndactyly syndrome (GCPS), which does not involve an anal phenotype (Johnston *et al.*, 2005, Wang *et al.*, 2000).

Hh activity is specifically required between 36 and 72 hpf for posterior gut development

The Smo antagonist, cyclopamine, was used to elucidate the temporal requirements for Hh signalling. Despite *ihha* being expressed from mid-somitogenesis and *shha* from 24 hpf, in the posterior gut region, cyclopamine treatment revealed that Hh pathway activity is not required before 34 hpf. This identifies distinct temporal requirements for Hh and BMP activity, as the latter is only required during early somitogenesis and the phenotypes of Hh and BMP impaired fish reflects this difference. The pronephric ducts, which initially share a common opening with the posterior gut in the presumptive cloaca are largely unaffected by a loss of Hh activity. This suggests that the defects are not due to mis-specification of the cloaca, and are distinct from the BMP dependent malformations described by Payati *et al.,* 2006), which cause cyst-like swellings in the pronephric terminus as well as posterior gut atresia. Consistent with this the *Hh* mutant fish have normal *bmp4* expression in the proctodeum and *prdm1* and *evx1* – both of which are altered in the *bmp* deficient zebrafish – are also unaffected by reductions in Hh activity. The temporally controlled exposure to cyclopamine supports the conclusion that it is the loss of Hh signals within the posterior gut, and not non-specific changes in the embryo overall, that cause the ARMs seen in both cyclopamine treated fish and the Hh pathway mutants. Treatment with cyclopamine after 24 hpf does not cause any curling of embryos typical of Hh mutants but does cause severe ARMs, ruling out the possibility that the latter is a secondary consequence of curling. Conversely, the relatively normal development of the posterior gut in embryos that are otherwise severely malformed following cyclopamine exposure prior to 24 hpf demonstrates its independence of other Hh regulated processes.

The zebrafish as a model for ARMs

ARMs are a significant clinical problem. The incidence is relatively high and can require extensive surgery to provide a tolerable quality of life. Even with surgical intervention the outlook for those with more serious defects is poor and for those who respond well to surgery there is often a long-term follow up required, in addition to psychological damage (Bai *et al.*, 2000, Hartman *et al.*, 2004). Our studies provide new insights into the molecular bases of ARMs, and suggest a robust model system for future analysis of the genetic and teratogenic contributors to ARMs.

Materials and Methods

Fish maintenance

Zebrafish were raised and maintained in compliance with UK Home Office guidelines using standard laboratory practice at 28.5ºC (Kimmel *et al.*, 1995). Adult fish identified as heterozygous for specific mutant alleles were inter-crossed to generate homozygous progeny. Embryos were harvested synchronously; staging based on developmental time was verified by morphological features as described by Kimmel *et al.*,1995). Lines used included the mutants: *sonic you* ^{t4}, *syu* ^{bx392} and *syu* ^{bq70} *(shha)* (Schauerte *et al.,* 1998), (van Eeden *et al.,* 1996); *smoothened* ^{b577} *(smu)* (Barresi *et al.,* 2000); *dtr (gli1)* (Karlstrom *et al.,* 1996, Karlstrom *et al.,* 2003); *yot* ^{ty119} *(gli2)* (Karlstrom *et al.*, 1999, Karlstrom *et al.*, 1996, Karlstrom *et al.*, 2003); *ihha* ^{hu2131} (ZF models); *spadetail (tbx16)* (Griffin *et al.,* 1998); *no tail* (*ntl, Brachyury* homolog) (Kimmel *et al.*, 1989, Odenthal *et al.*, 1996); *doc* (gene unknown) (Odenthal *et al.*, 1996); *bashful (lama1)* (Karlstrom *et al.*, 1996); *sleepy (lamc1)* (Karlstrom *et al.*, 1996), (Parsons *et al.*, 2002) and *crash test dummy* (*ctd,* gene unknown) (Odenthal *et al.*, 1996); *floating head (flh,* a *not* homeobox gene) (Odenthal *et al.*, 1996), (Talbot *et al.*, 1995) .

Live imaging

Live embryos were anesthetised with Tricaine (MS222; pH7.0) and immersed in 3% methylcellulose or in fish water and observed using a Zeiss Axioplan microscope. Images were acquired using a SPOT 14.2 colour mosaic camera (Diagnostic Instruments) and processed using Photoshop software (Adobe).

Immunohistochemistry

Embryos were whole-mount stained with 0.25 μM FITC-phalloidin (Sigma) and propidium iodide (Vector; 1:500; 1mg/ml stock), mounted in Vectashield (Vector) and imaged with a Leica SP confocal microscope.

Fluorescein injections

0.3 μg/ml fluorescein salt (Sigma 28803) was injected into the pericardial sac of anesthetised embryos between 48-96 hpf. Development was allowed to proceed for 24 hours. Accumulation and excretion of salt in and from the gut lumen was detected under a fluorescent microscope using a Hamamatsu C4742-95 camera and Open Lab software (Improvision).

TUNEL

Embryos were fixed for TUNEL staining at 24 hpf, 48 hpf, 72 hpf, 96 hpf and 120 hpf in 4% paraformaldehyde for two hours at room temperature. TUNEL was performed as described in the manufacturers protocol (Intergen).

Cyclopamine treatment

Embryos were treated with cyclopamine as previously described (Chen *et al.*, 2001). In summary cyclopamine (Toronto Research Company) was dissolved in 95% ethanol to generate a 10 mM stock solution and further diluted in embryo media to the appropriate working concentration. Three 10 minute washes in 50 ml of PBS were used to remove cyclopamine from embryos when necessary before fixing, photographing or allowing development to proceed.

Whole-mount in situ *hybridisation*

In situ hybridisation was performed as previously described (Begemann and Ingham, 2000). Probes used have been described previously: *shha* (Krauss *et al.,* 1993), *ihha* (Qiao, 1996) *ptc1* (Concordet *et al.,* 1996), *gli2a* (Karlstrom *et al.,* 1999), *bmp4* (Hwang *et al.*, 1997), *prdm1* (Baxendale *et al.*, 2004) and *evx1* (Thaeron *et al.*, 2000).

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