Identification, visualization and clonal analysis of intestinal stem cells in fish

Narges Aghaallaei* ‡1, Franziska Gruhl*1,2, Colin Q. Schaefer1, Tobias Wernet1,3, Venera Weinhardt1,3, Lázaro Centanin1, Felix Loosli3, Tilo Baumbach3, and Joachim Wittbrodt‡1

*Authors contributed equally to this work
‡ Authors for correspondence
1: Centre for Organismal Studies, COS, Heidelberg University, Heidelberg, Germany
2: Present address: Computational Biology at UNIL, Lausanne, Swiss
3: Karslruhe Institute for Technology, KIT, Karlsruhe, Germany
Abstract

Recently, a stochastic model of symmetrical stem cell division followed by neutral drift has been proposed for intestinal stem cells (ISCs). This division mode has been suggested to represent the predominant mode of stem cell progression in mammals. In contrast, stem cells in the retina of teleost fish show a preferential asymmetric division mode. To address whether the mode of stem cell division is following phylogenetic or ontogenetic routes we characterized and analysed the entire gastrointestinal system with a particular focus on ISCs in the teleost medaka (Oryzias latipes). We analysed the entire intestine in adult medaka by X-ray micro-computed tomography within the intact body and combined that analysis with the cellular and molecular composition of the intestinal system. This allowed correlating the 3D topography with functional domains of the intestinal system. Morphology and gene expression data consistently argue for a subdivision of the medaka intestine into a small and large intestine. Analysis of ISCs in proliferation assays and via genetically encoded lineage-tracing highlights a stem cell niche in the furrow between the long intestinal folds. This niche is functionally equivalent to the mammalian intestinal crypts and stem cells in this compartment are characterized by the expression of sox9, axin2 as well as lgr5, homologs of mammalian ISC markers, emphasizing the evolutionary conservation of the Wnt-pathway components in the stem cell niche of the intestine.

Consistent with a preferential symmetric division mode of medaka ISCs, the stochastic, sparse initial labelling of ISCs ultimately resulted in extended labelled or unlabelled domains originating from single stem cells in the furrow niche contributing to both, homeostasis and growth. Thus different modes of stem cell division co-evolved within one organism, and in the absence of physical isolation in crypts, ISCs contribute to homeostatic growth.
Introduction:

Recent key findings have stimulated the debate about the division mode of adult stem cells (symmetric vs. asymmetric) in mammals in particular and vertebrates in general. While on the one hand prominent symmetric cell divisions followed by neutral drift are best explaining the division of multi-potent ISCs in mouse (Snippert et al., 2010), the preferential asymmetric division mode of multi-potent stem cells is driving functional homeostasis during retinal growth in the teleost retina (Centanin et al., 2014). This poses the question whether there is an ancestral (asymmetric) and derived (symmetric) mode of stem cell progression. Alternatively, and independent of the phylogenetic position of the organism, stem cells could divide by symmetric or asymmetric division in a niche specific manner. The answer to that may shed light on the interplay between the presence of stem cells and the formation of tumours. Adult stem cells with self-renewing properties (e.g. ISCs) are crucial for tissue homeostasis and closely resemble the properties of tumour cells. The well-described, predominant asymmetric division mode of teleost retinal stem cells allows comparing how stem cells behave under conditions of organ growth and tissue homeostasis in the life-long growing intestine.

The basic vertebrate bauplan of the digestive tract in fish shows adaptations related to phylogeny, ontogeny, environment and diet of each species (Grosell et al., 2010). Along the rostro-caudal axis it is divided into three segments: The rostral intestinal bulb, the mid-intestine and the caudal intestine (Wallace et al., 2005; Wang et al., 2010). Microarray analyses of the expression of metabolic genes in the adult zebrafish gut suggest a subdivision into small and large intestine (Wang et al., 2010). Similar to the mammalian intestine, the inner layer of the intestine is folded in many fish species, although intestinal folds are often less distinct, while villi and crypts as in mammals are not described for medaka or zebrafish.

In mammals, ISCs drive homeostasis by the continuous replacement of the intestinal epithelium. This renewal is species specific and takes between two and seven days (Clevers, 2013a; Creamer et al., 1961; Crosnier et al., 2006). Gene targeting and lineage tracing studies in mouse revealed that ISCs residing in the intestinal crypt base of the small intestine divide and the descendants are pushed towards the villus tip where they exfoliate (Barker et al., 2007; Clevers, 2013b; Ritsma et al., 2014; Tan and Barker, 2014; Tian et al., 2011; Vermeulen and Snippert, 2014). In intestinal homeostasis and for the maintenance of
ISCs, the Wnt pathway plays a key role (Clevers et al., 2014; Fevr et al., 2007; Korinek et al., 1998). Among the Wnt target genes, Lgr5 is to date the most important marker for stem cells of the small intestine and colon (Barker et al., 2007). Lineage tracing experiments in mouse revealed that Lgr5 or Bmi1 expressing ISCs can repopulate entire intestinal crypts (Barker et al., 2007; Sangiorgi and Capecchi, 2008). The high mobility group box transcription factor Sox9 is another Wnt target gene regulating cell proliferation in the intestine (Bastide et al., 2007; Blache et al., 2004). Its loss of function affects differentiation throughout the intestinal epithelium and results in the loss of Paneth cells (Bastide et al., 2007), which provide important niche factors to keep ISCs in their proliferative state (Sato et al., 2011).

In the lifelong growing fish intestine, so far, a domain of proliferating epithelial cells was reported at the base of the intestinal folds (Rombout et al., 1984; Stroband and Debets, 1978; Wallace et al., 2005). However, the molecular setup of these epithelial cells was not addressed so far.

To ultimately compare the mode of stem cell division in the growing retina with the stem cell division during homeostasis and tissue growth in the intestine of medaka, we analysed the intestine by high-resolution X-ray micro-computed tomography (micro-CT), histochemistry and gene expression studies and the characterization of ISCs with molecular, genetic and lineaging tools. We show key morphological and molecular features such as the division into a large and small intestine, the presence of folds and the distribution of proliferative and apoptotic cells along the folds of the medaka intestine. Importantly, we identify a proliferative compartment at the furrows between the intestinal folds that in many respects resembles the mammalian stem cell niche in the intestinal crypts. These cells express homologs of the mammalian ISC markers including lgr5, which was not reported in the fish so far. Our lineage analysis data are consistent with a predominantly symmetric mode of stem cell division in the intestinal furrows of the medaka intestine resulting in homeostatic growth.

Taken together our data indicate that the mode of division of particular stem cells is not a species-specific feature but rather a feature of the specific stem cell type, rather correlated with the function of the stem cells in tissue homeostasis and/or organ growth.
Material and Methods

Fish

Medaka (*Oryzias latipes*) stocks were maintained as previously described (Koster et al., 1997). All fish (Icab inbred strain) are maintained as closed stocks at the Centre for Organismal Studies (COS) at Heidelberg University and the Institute of Toxicology and Genetics of the Karlsruhe Institute of Technology (KIT). Fish husbandry and experiments were performed according to local animal welfare standards (Tierschutzgesetz 111, Abs. 1, Nr. 1, Haltungserlaubnis AZ35-9185.64 and AZ35-9185.64/BH KIT) and in accordance with the European Union animal welfare guidelines. The fish facilities are under the supervision of the local representative of the animal welfare agency. Embryos were staged according to (Iwamatsu, 2004). If not stated differently, adult fish were defined as at least three months old which are under normal laboratory conditions sexually mature. The *sox9b:EGFP* transgenic fish was described previously (Nakamura et al., 2008).

X-ray micro-CT imaging

Seven-week-old medaka were sacrificed, fixed and imaged at the TOPO/TOMO beamline at ANKA, the synchrotron radiation facility of the KIT. Fish were sacrificed according to the German animal welfare act and immediately fixed (4% formaldehyde, 1% glutaraldehyde for three days at room temperature). For better X-ray contrast, fish were stained by phosphotungstic acid (0.3% phosphotungstic acid in 70% ethanol for three days). Samples were washed, embedded in agarose, sealed in polypropylene containers and mounted on a standard tomographic table. We used a parallel, monochromatic beam setup (Double-Multilayer Monochromator, 2% energy bandwidth, energy set to 16 keV) with an additional Al filter (0.2 mm). The optical setup consisted of a LAG scintillator, converting photons to the visible light spectrum, a magnifying microscope setup (total magnification 3.6x) and a CMOS camera (pco.edge, 2560 x 2160 pixels, 6.5 x 6.5 µm² pixel size), resulting in an effective pixel size of 1.81 µm. For each sample we recorded a full 360° tomographic scan of 1500 projections. MATLAB was used for image processing of raw data, unless stated otherwise. PyHST algorithm (Chilingaryan et al., 2011) was used for 3D data reconstruction. Those data were denoised with a non-local means filter for further analysis. Detailed...
protocols and scripts are available on request. General image processing was done with Fiji/ImageJ (Schindelin et al., 2012). Segmentation of the gut was performed by edge-based and region competition snake algorithms with the ITK SNAP software (Yushkevich et al., 2006). 3D rendering for visualization and 3D structure analysis was performed with Amira and ImageSurfer2 (Feng et al., 2007).

RT-PCR

The gut tubes of three adult medaka females were cut into six equal pieces. Total RNA was extracted from each piece using the TRIzol reagent (Ambion) according to the manufacturer instructions. 1µg of RNA was used to synthesize cDNA with the Superscript\textsuperscript{TM} First-Strand Reverse Transcriptase kit (Invitrogen) and oligo (dT) primer. Primers for RT-PCR are listed in Table S1. For PCR analysis the Q5 High-Fidelity DNA polymerase Kit from BioLabs was used. PCR conditions: denaturation 98°C, 30 seconds; then 30 cycles at 98°C, 10 seconds; annealing: 57°-60°C, 30 seconds; 72°C, 30 seconds; final extension 72°C, two minutes.

Isolation of lamprey and medaka probes

*axin2a, axin2b, rspo1, rspo3, lgr4, lgr5* and *lgr6* genes were amplified from medaka adult gut cDNA. *aldh1a2, ascl1a, bmi1, bmi3, sox9b* was isolated from a medaka cDNA library (Souren et al., 2009). Amplified DNA fragments were cloned into the pGEM-T easy vector (Promega) and sequenced validated. Primers for PCR and library IDs are listed in Table S2. Linearized DNA was *in vitro* transcribed with Sp6 or T7 polymerase according to the standard protocol for generation of the antisense probes. cDNA of lamprey was provided by the laboratory of T. Boehm. Primers listed in Table S2 were designed considering the *Petromyzon marinus* Sox9 protein sequence (Accession number: DQ136023.1) and an alignment with Japanese lamprey genome.
**In situ hybridization**

*In situ* hybridization on paraffin sections was performed with digoxigenin (DIG)-labelled antisense probes as described (Aghaallaei et al., 2005). Images of RNA in *situ* hybridization were taken with a Zeiss Axio Imager.

**EdU proliferation assay**

Six-week-old fish were incubated in 100µM 5-ethyl-2'-deoxyuridine (EdU) for 24hrs. Fish were fixed in 4%PFA/2xPBST after 12, 36, 50 and 122hrs. After fixation, fish were embedded in paraffin according to standard protocols. EdU detection on sections (7-9µm) was performed using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Invitrogen) as described previously (Bajoghli et al., 2011). Fluorescence microscopy was performed with a Leica TCS SPE, equipped with a 40x oil objective and AxioVision software. The proliferation assay in lamprey was done as described (Bajoghli et al., 2009) by injection of EdU (5µg). EdU proliferation assays were performed at EMBL.

**KNIME**

Quantification of cell proliferation in the intestine was performed using the KNIME image processing toolbox (KNIP). Segmentation of DAPI-stained nuclei was performed using the KNIP spot detection node. The folds were divided into four equally high trapezoids along the apical-basal axis. Total number of epithelial cells as well as number of EdU positive cells in each of the four trapezoids was determined. Further details and the respective KNIME workflow are available on request.

**Lineage tracing, image acquisition and analysis**

For lineaging, medaka Gaudi lines (Centanin et al., 2014) were used. Cre-recombination with tamoxifen-induction (5-10µ, TM5648 Sigma) was performed for 3hrs at 12 dpf (stage 40). In case of heat shock induction, embryos were incubated at 39°C for two hours and fixed 90 days post induction (dpi). After 1-10-30-150 days respectively, fish were sacrificed, fixed in 4%PFA/2xPBST o/n at 6°C, and divided into two groups. One group was cryopreserved in 30% sucrose, embedded in Leica tissue freezing medium and sectioned
at 30-50µm on a cryostat. Confocal images of the sections were collected using a Leica SPE and processed using the Leica application suite X software (LASX) and Adobe Photoshop CS4. From the other group, guts were dissected and used for whole gut imaging with a NikonSMZ18 microscope and Luxendo GmbH (Heidelberg) 25x MuVi-SPIM. In general, image processing was conducted with Fiji (Schindelin et al., 2012; Schneider et al., 2012). For 3D reconstructions Imaris software was used. Surfaces of clones and the according movies were rendered with Chimera from UCSF (Pettersen et al., 2004). Raw data movies of GaudiLxBBW were rendered with Vaa3d (Peng et al., 2014). Quantification of clone size was conducted by thresholding for nuclei and measuring clone volume with the BoneJ Command “Particle Analyser”(Doube et al., 2010). This clone size was divided by a standard size of cell nuclei found in the data (4.4 µm³) and rounded down to next full number. Subsequently, clones consisting of no cells were discarded as artefacts. For statistical analysis and visualization of the data, GraphPad Prism version 7.00 for Windows and Mac OS X, GraphPad Software, San Diego California USA, www.graphpad.com was used.

**Immunohistochemistry and H&E staining**

Immunostaining and H&E staining were performed as described previously (Inoue and Wittbrodt, 2011). Nuclear DNA was stained with DAPI or DRAQ5 (Smith et al., 2004).

**Phylogenetic analyses**

Protein sequences of various vertebrate LGRs were retrieved from NCBI: *Danio rerio* LGR4 (Accession No. E7FE13), *Danio rerio* LGR6 (Accession No. P0DM44), Human LGR4 (Accession No. AAH33039), Human LGR5 (Accession No. AAH96325), Human LGR6 (Accession No. AAH47905), Human LGR7 (Accession No. AAG17167), mouse LGR4 (Accession No. NP_766259), mouse LGR5 (Accession No. NP_034325), mouse LGR6 (Accession No. NP_001028581), *Oryzias latipes* LGR4 (Accession No. BAM29306), *Oryzias latipes* LGR5 (predicted, Accession XP_004085681), *Oryzias latipes* LGR6 (Accession No. BAM29305), Xenopus laevis LGR5a (Accession No. ADK66918), Xenopus laevis LGR5b (Accession No. ADZ55458). BLAST at the ENSEMBL (http://www.ensembl.org/info/about/species.html) confirmed identity of medaka lgr5 (*Oryzias latipes*, scaffold2947: 4,002-5,617). Alignments were created with ClustalW and phylogenetic relationships were deduced after alignment of protein sequences with
Results and Discussion

Morphology of the medaka gut

To characterize the overall morphology of the adult medaka gut with subcellular resolution, we used a micro-CT station at the TOPO/TOMO beamline (Rack et al., 2009). X-rays penetrate thick and opaque tissues to provide a complete three-dimensional image of large samples in toto without the need of sectioning. We recorded and segmented an in toto perspective of the gut of a young adult medaka. This 3D view reveals three distinct topographic domains along the rosto-caudal axis of the intestinal tract (Movie1-2): The buccal cavity (mouth), the esophagus and the intestine, latter characterized by varying shapes from anterior to posterior (Fig. 1A, Movie1-2). We noticed a marked difference in the cavity of the anterior intestine in comparison to the posterior intestine. The bile duct, connecting the gall bladder with the anterior part of the intestine (ductus choledocus, Fig. S1A) marks a position equivalent to the duodenum in mammals. The inner wall of the gut in medaka is wrinkled into structures protruding into the lumen (folds). The lumen size and the density and extent of folds are decreasing along the rosto-caudal axis (Fig. 1B-E).

To assess the morphology of the epithelium in higher detail, we applied hematoxilin & eosin staining on histological transverse-sections of seven-week-old young adult fish. The buccal cavity contains papillae, formed by high prismatic epithelial cells containing a large number of the mucus secreting goblet cells (Fig. 1F and 1J). The esophageal mucosa is folded into ridges that are strongly surrounded by muscles (Fig. S1B-C). The epithelium is stratified with numerous intra-epithelial aggregates of mucus secreting goblet cells (Fig. S1D-E). This high number of mucus-secreting cells facilitating the flow of food towards the intestine. The prismatic cells that form the intestinal epithelium rest on connective tissue containing blood vessels and muscle fibres, similar to the lamina propria of the mammalian intestine (Fig. 1J-M).

Folds in the anterior intestine and the midgut are densely packed and display an elongated, ridge like shape (Fig. 1H,L). The number of folds decreases towards caudal. They broaden, get shorter and are almost absent close to the anus (Fig. 1F-M). In mammals, ISCs reside in crypts of Lieberkühn of the intestine. We did not identify analogous invaginations
in the medaka intestine. Columnar-shaped enterocytes are the most prominent cell type of the intestinal epithelium followed by the mucus-secreting goblet cells, present in all intestinal domains (Fig. 1J-M). Altogether our morphological analyses show that the medaka and mammalian digestive tract share a number of features.

**Subdivision of the medaka gut into a small and large intestine**

To examine whether the morphological domains of the intestine correlate with distinct gene expression domains, we analysed specific marker genes for the small and large intestine present in the respective structures of the mammalian intestine. We divided the gut of a young adult medaka (buccal cavity and oesophagus were not included) into six segments (S1 to S6) and addressed the expression of the intestinal marker genes in each segment by semi-quantitative RT-PCR. As markers for the small intestine, we employed the apolipoprotein apoa1 as well as the fatty acid binding proteins fabp2 and fabp6. Apoa1 is a key player in cholesterol homeostasis and is highly restricted to the digestive organs including the small intestine (Wang et al., 2010). Consistently, medaka apoa1 was detected in the first four gut segments (Fig. 1N-O). In mammals, Fabp2 is involved in the uptake and intracellular transport of fatty acids in the small intestine (Chmurzynska, 2006). Like apoa1, medaka fabp2 was expressed only in the first four gut segments (S1-S4). In mammals, Fabp6 acts as an intracellular transporter of bile acids in ileal epithelial cells (most distal part of the small intestine), helping to catalyse and to metabolize cholesterol (Praslickova et al., 2012). In medaka, fabp6 expression was detected in the segments S3-S6, with a very weak expression in S1 and S2 (Fig.1N-O). Cathepsin L1 (ctsl1) is a marker for the colon-like intestinal region in zebrafish (Wang et al., 2010) and is found in the corresponding domain in medaka (S5 and S6).

The expression profiles of the marker genes as well as the histological features of the medaka intestine indicate that the segments S1-S4 of the medaka intestine resemble the mammalian small intestine. This stretch comprises long and densely packed folds and exhibits, with the expression of apoa1 and fabp2, a mammalian small intestinal fingerprint. The low expression of ctsl1 as well as expression of fabp6 in the segments S3/S4 indicates a distinct transition zone between small and large intestine. The remaining segments S5 and S6 of the medaka intestine comprise large intestine characteristics, as indicated by broader and fewer folds and the strong presence of the colon markers ctsl1 and fabp6.
**Expression of ISC markers in the intestinal furrows**

The high the morphological similarity between the medaka and the mammalian intestine likely extends into the molecular mechanisms controlling ISCs. In the murine intestine several signalling pathways, including Wnt, Notch, BMP (bone morphologic protein) and Hedgehog orchestrate epithelial homeostasis (Sancho et al., 2004). Lgr5, member of the family of G-protein-coupled receptors, represents a Wnt target gene and is a prominent marker for ISCs residing at the base of the crypts (Baker et al., 2015; Barker et al., 2007; Clevers et al., 2014). While lgr4 and lgr6 have been described in zebrafish (Hirose et al., 2011) and medaka (Deguchi et al., 2012), there are no reports of lgr5 expression in fish. We identified the orthologue of lgr5 in the medaka genome and analysed its expression together with the expression of additional ISC marker genes in the intestine of young adult medaka (Fig. 2). We found that the expression of lgr5 and its paralogs lgr4 and lgr6 is confined to the intestinal furrows in the medaka small intestine, where lgr5 is confined to the furrow base (Fig. 2A-C).

We used the expression of the polycomb complex protein bmi1, a marker for quiescent ISCs in mouse (Sangiorgi and Capecchi, 2008; Yan et al., 2012), to identify the equivalent cells in medaka. Here, a weak expression of bmi1 and bmi3 was detected at the base and the mid-base segments (Fig. 2D,E).

In addition, we investigated the expression several other marker genes specific for ISCs or sub-structures of the mammalian intestinal crypt. We detected expression of the aldehyde dehydrogenase aldh1a, a marker for normal and cancer stem cells (Huang et al., 2009) in the connective tissue underlying the intestinal furrows (Fig. 2F).

The gene ascl1a (Transcription factor achaete scute-like) an orthologue of the mammalian transcription factor and intestinal stem cell marker Ascl1 (Rizk and Barker, 2012; van der Flier et al., 2009) is expressed throughout the entire fold with sporadic elevated levels in single cells in the intestinal furrow (Fig. 2G).

R-spondins enhance low-dose Wnt signals (Kazanskaya et al., 2004) and Rspl stimulates crypt stem cell proliferation in mouse (Kim et al., 2005). We detected expression of the medaka rspolat at the base of the intestinal furrow (Fig. 2H-I). Rspl3 is not expressed in the fold itself, but in cells of the underlying connective tissue similar to aldh1a (Fig. 2I).

Axin is an important Wnt suppressor (Ikeda et al., 1998). The medaka paralogs axin2a and axin2b were detected in intestinal epithelial cells, where axin2b was more restricted to the
base of the intestinal furrow (Fig. 2J,K).

Of all marker genes highlighting ISCs, the transcription factor Sox9 showed the most confined expression pattern. In mouse, distinct levels of Sox9 are expressed in stem and differentiated cell populations of the small intestinal epithelium (Formeister et al., 2009; Furuyama et al., 2011; Gracz et al., 2010; Van Landeghem et al., 2012). Schartl and colleagues had identified Sox9 paralogs (sox9a, sox9b) in teleosts (Kluver et al., 2005) and had shown that only sox9b is expressed in the distal intestine of late embryos and early juveniles. In our analysis of the adult medaka intestine, sox9b expression is confined to a small number of cells at the base of the intestinal furrow (Fig. 2L). Taken together, the consistent localized expression of ISC marker genes at the intestinal furrow of the adult medaka intestine hints at this domain harbouring ISCs of medaka. Our analysis is consistent with the idea that the molecular mechanisms underlying ISC proliferation in fish resemble those proposed in mouse.

**Sox9b is a putative marker for proliferative progenitor and ISCs in medaka**

The transcription factor Sox9 is required for the induction and maintenance of several types of vertebrate stem cells including ISCs (Formeister et al., 2009; Furuyama et al., 2011; Gracz et al., 2010; Van Landeghem et al., 2012). The conspicuous expression of sox9 in different models including Xenopus (Lee and Saint-Jeannet, 2003), chicken (Shyer et al., 2015) and mouse (Blache et al., 2004) hints at an essential role of this gene in the gut. Even in the larval gut of the basal vertebrate lamprey (*Lampetra planeria*) we identified sox9 expression in proliferating cells (described previously (Bajoghli et al., 2011) at the base of the typhlosole (Fig. 3A-B), a longitudinal fold of the inner intestinal wall (Fig. 3C), hinting at its evolutionarily conserved function (Fig 3).

In medaka, expression of the mammalian sox9 orthologue (sox9b) was reported in the gonads (Kluver et al., 2005; Nakamura et al., 2010). We show that sox9b is also expressed in the intestine, confined to the furrow between the intestinal folds. To correlate expression and proliferative capacity of sox9b+ cells we employed transgenic fish expressing eGFP under the control of the sox9b regulatory elements (Nakamura et al., 2008). Here, eGFP expression is restricted to a few intestinal epithelial cells located at the furrow between the intestinal folds at larval stages (10 dpf), three-week-old juvenile and eight-week-old adult fish (Fig. 3D-F).
To test whether cells expressing *sox9b* actively proliferate, we assayed EdU incorporation in *sox9b*+ cells and co-detected EdU and *sox9b* by whole mount *in situ* hybridization or in the transgenic line. Twelve hours after a 24hrs EdU pulse, *sox9b*+ cells co-localize with EdU positive cells (Fig. 3G) in young adults. We conducted a comparable EdU pulse-chase experiment in one-month-old fish in the *sox9b:eGFP* transgenic line. Also in those fish, cells expressing *sox9b* had incorporated EdU 24hrs after the EdU pulse (Fig. 3H, n=3 fish, 63 cells), indicating the proliferative activity *sox9b*+ cells in the juvenile and adult medaka intestine. To finally address the stem cell nature of *sox9b*+ cells, we addressed *Sox9b* expression initiator cells of a clonal lineage. We analysed *sox9b* in the Gaudi transgenic line (Centanin et al., 2014) initiating lineaging by stochastic activation of ubiquitin$^{ERT2}\text{Cre}$ in Gaudi$^{RSG}$-fish by tamoxifen treatment (10µM, 3hrs) at stage 40 of development. Two weeks after induction we correlated lineage traces and *sox9b* transcripts by confocal microscopy (Fig. 3I). All GFP positive lineages analysed (n=66 lineages in 4 fish) co-localised with *sox9b* expression at their point of origin in the proliferative zone at the base of the intestinal furrows. Thus *Sox9b* represents an evolutionarily conserved marker for proliferative progenitor and ISCs.

**Proliferative cells are predominantly located in furrow between intestinal folds**

It has been proposed in previous studies in different fish species that compartments of proliferating epithelial cells are located at the base of the intestinal folds (Faro et al., 2009; Hellberg and Bjerkas, 2000; Rombout et al., 1984; Wallace et al., 2005). To identify the position of proliferating cells and ISCs in the medaka gut, we stained mitotically active cells immunohistochemically detecting phosphorylated histone H3 (pH3) and EdU incorporation respectively. We subdivided the intestinal folds from basal to apical into four equally sized segments: Base (B), middle-base (MB), middle-tip (MT) and tip (T) and determined the relative number of pH3+ and EdU+ cells in each of the segments (Fig. 4A). Analysis of cells in M-phase revealed that the majority of pH3 positive cells (85.71%, nFolds = 20) is located in the basal half of the folds (B + MB). Only few proliferating cells were found in MT (14.9%, nFolds = 20) and none in T (Fig. 4A). We resolved the temporal dynamics of intestinal proliferation in an EdU pulse-chase assay. Young adult fish were incubated in EdU for 24hrs and were sacrificed 12, 36, 50 and 122hrs post-treatment. The number of EdU+ cells was counted in each segment of the folds at the time point indicated (Fig. 4C). This revealed a progression of initially labelled cells from the basal sector to the tip of the folds. The number
of EdU+ cells remained always highest at the base of fold, consistent with the stem cell niche being located in the furrow between the folds. The increase of EdU+ cells at the tip (T) from 4.6%±1.5 at 12hrs post-incubation to 14.0%±4.3 at 120hrs post-incubation (Fig. 4D) reflects the flux of cells from base to tip. Apoptotic cells only present at the tip of the fold indicates the shedding of intestinal cells in this segment (Fig. 4B). Similar experiments in zebrafish had determined a turnover time 7-10 days for the base to tip transition (Wallace et al., 2005).

Taken together, our results identify a population of mitotically active cells located at the base between intestinal folds. EdU+ cells are initially found at the base and are subsequently shifted upwards to the tip. Our data in medaka indicate that it takes between five and up to ten days from the birth of intestinal cell types at the base to apoptosis at the fold summit, where cells are shed off into the intestinal lumen.

**Identification of ISCs in the furrow between the intestinal folds**

To ultimately identify and trace ISCs and their division mode we performed long-term lineage analysis in the medaka intestinal folds during life-long organ growth and homeostasis. This not only allowed following the lineage of individual cells, but also addressed the mode of stem cell division in the niche. To stochastically label individual cells in the medaka intestine we used the brainbow-based (Livet et al., 2007) Gaudí toolkit (Centanin et al., 2014). We combined the two-color fluorescent reporter line Gaudí$^{RSG}$ (Gaudi Red-Switch-Green) with ubiquitin$^{ERT2}$Cre, a line that allows stochastic activation of Cre recombinase by tamoxifen induction. The timeline of the experiment is presented in Figure 5A. Tamoxifen applied at late embryonic stages (d12, Fig. 5A) triggers the stochastic recombination in individual cells in the intestine of the Gaudí$^{RSG}$ line, resulting in a switch from red to nuclear green in recombined cells and all of their descendants. Tamoxifen concentration (5-10µM) and duration of the treatment (three hours) were adjusted to achieve a labelling of clearly discernible, individual cells within the intestine. After stochastically triggering permanent H2B-EGFP expression, eventually only mitotically active ISCs will contribute to continuous strings of labelled cells. Only, if the initial recombination occurred within a stem cell, it will retain its label and will transmit it to all of its descendants. Conversely, recombination in progenitor or differentiated cells will result in the transient
labelling of small clones or individual cells. The continuously replacement of epithelial cells in the intestine will ultimately only permit the detection of labelled ISCs and their clonally continuous descendants (Fig. 5B-E’’).

In a scenario of predominantly asymmetric stem cell divisions, the total number of clonal strings originating from a single stem cell will remain constant. Symmetric division and neutral drift conversely, will result in a marked decrease of the number stem cell clones on one hand, while at the same time the clone size will increase.

We investigated the development of stem cell clones over time in three complementary approaches (Fig. 5B-E’’) and quantified the density, distribution and number of labelled cells/clones (Fig. 5F-H) in two independent experimental settings with 5 to 20 individuals per time point analysed.

Imaging whole mount preparations of dissected intestines at different time points after clonal induction provides a qualitative impression of the distribution and development of the labelled cells (Fig. 5B-E). The initial labelling of many discrete clones composed of individual cells at the first dpi (Fig. 5B) is strongly reduced over time (Fig. 5C, D) and is eventually (150dpi) confined to a few clones of enormous size (Fig. 5E). We analysed intestinal sections (30-50µm) of the same fish by confocal microscopy (Fig. 5B’-E’). This analysis, as shown in a 3D representation of the cross sections, confirms the gross morphological analysis in Fig. 5B-E. Initially, many small clones are evenly distributed (Fig. 5B’). Eventually only few remaining clones extending from the base to the top of the intestinal folds increase in size along the longitudinal axis of the ridge (visible 10dpi, Fig. 5C’, and prominent 30 and 150dpi respectively, Fig. 5D’, E’).

The distribution of labelled clones was quantified (Fig. 5F) in cross sections of the small intestine (five to 19 animals per time point, on average 7 distantly spaced sections per animal) by relating the number of GFP containing labelled folds to unlabelled folds in the same section. An initially high range of positive cells distributed at 1dpi is narrowed down at subsequent time points after induction. The relative distribution per section is strongly reduced from 75% at 10dpi to 19% at 150dpi reflecting a 3.9 fold decrease in density (Fig. 5F).

In a third approach we imaged and counted labelled cells and clones in a large volume of the intestine (532x532x532µm) using light sheet imaging of the recombined intestines at identical time points after induction (Fig. 5B’’-E’’), G-H). This analysis highlighted the longitudinal expansion of the clones (growth along with homeostasis) and unambiguously unveiled the symmetric mode of stem cell division. After the initial stochastic labelling of
individual cells (1dpi, Fig. 5B’’), symmetric division and neutral drift established larger, clonally related groups of stem cells (10dpi, Fig. 5C’’, movie 3) eventually resulting in intestinal folds that are either broadly labelled by H2B-EGFP, or not at all (Fig. 5D’’, E’’, movie 4-5).

With the full 3D dataset of the MuVi-Spim we quantified clone number, cell number within individual clones and corresponding clone size at three time points after clone induction (10dpi= 693 clones, 4 intestinal segments; 30dpi=178 clones, three intestinal segments; 150dpi=35 clones, 15 intestinal segments, Fig. 5G-H). In brief, we determined clone volume by thresholding of the nuclear H2B-GFP and determined the number of cells per clone by division by the average nuclear volume (details in materials and methods). These analyses show an increase in clone size from one cell to 1600 (on average) cells from 10dpi to 150dpi, while at the same time, clone numbers decrease from 1000 to 12 clones per mm³ (Fig. 5E’, E’’, H, movie 5). The quantification of labelling distribution, cell numbers and clonal density underpins the visual impression and strongly supports symmetric cell division of ISC s followed by neutral drift to contribute to homeostatic growth.

Taken together, our qualitative and quantitative analyses revealed a clear prevalence of symmetric stem cell divisions in the medaka intestine, analogous to the findings in the murine intestine and different from the divisions mode of stem cells in the retina. Symmetric cell division and neutral drift ultimately result in large mono-clonal domains of the intestinal epithelium. These domains expand widely in the furrow between the folds (Fig. 5E’’, movie5) and are eventually composed of hundreds to thousands of stem cells (Fig. 5E,E’’,E’’,G), contributing to both adjacent folds, arguing for a joint niche for both flanks. Clearly, the intestinal folds physically restrain the stem cells to the furrow. Consequently, each fold is composed of two flanks of separate origin in the respective furrows (Fig. 5D’’-E’’, movies3-5).

To address whether the large domains forming are not due to the fusion of initial clusters of stem cells we stochastically triggered clonal multi-colour labelling in the GaudilBBW line (Centanin et al., 2014) by a limited heat shock activating Cre recombinase via the hsp70:nlsCRE transgene (Fig. 6A). We analysed the resulting intestines after 90 days of homeostatic growth by MuVi-Spim imaging and identified adjacent, clonal domains of different sizes, discernible by different fluorescent proteins and their corresponding localization (Fig. 6B-C, movie6, n=3, eight gut pieces). Taken together all our data consistently argue for individual stem cells undergoing symmetric cell divisions followed by neutral drift, which ultimately results in clonal expansion or shrinking (Fig. 5, 6). In extreme
cases, neutral drift can shift entire sections of the fold into a mono-clonal valley (movie5).

**Conclusion**

Our detailed study of the fish intestine has revealed a striking similarity to the mammalian intestine ranging from the overall structure to the molecular definition of specific intestinal domains. The entire intestine is in continuous growth and homeostasis and all differentiated cell types originating from ISCs are ultimately shed into the intestinal lumen (Fig. 7). ISCs reside in the furrows at the base of intestinal folds and express typical stem cell markers including lgr5 (Leushacke et al., 2013; Lim et al., 2013) that we discovered in the medaka gut. The comparative analysis of sox9 (Formeister et al., 2009) expression identifies a domain of proliferatively active cells not only in teleosts, but also in basal vertebrates (lamprey). The expression of sox9 in proliferating cells at the base of the intestinal fold suggest that the stem cell niche has evolved from a longitudinal, extended furrow in basal vertebrates to an isolated crypt in mammals (Formeister et al., 2009). The multiple folds and furrows in fish represent a transition state. Analysis of the mode of intestinal stem cell division in medaka revealed a predominant symmetric cell division, highly reminiscent of the situation in mammals (Snippert et al., 2010). One of the consequences of the symmetric division mode in mouse is that all intestinal cells in a crypt are eventually of monoclonal origin (Snippert et al., 2010). In the case of the fish intestinal stem cell niche, we uncovered a progressive lateral extension of a “mono-clonal” domain likely by neutral drift. This domain could, hypothetically, extend along the entire furrow between the intestinal folds. Even though neutral drift controls a monoclonal domain that can grow or shrink, there is no physical barrier to prevent its expansion along the entire intestinal fold. Not restraining longitudinal extension easily facilitates the coupling of tissue homeostasis and organ growth. The evolution of the intestine and the formation of crypts in birds and mammals ultimately ensures the maintenance of poly-clonality by physical isolation, a poly-clonality that is composed of individual, monoclonal units represented by the intestinal crypts (Rinkevich et al., 2014; Roy et al., 2014; Shyer et al., 2015). The evolutionary advantage of retaining poly-clonality of the intestinal epithelium of mammals and birds goes along with the uncoupling of tissue homeostasis from organ growth, which are still clearly coupled in fish. The cell division mode in the intestine is in clear contrast to the situation in the continuously growing fish retina, where stem cells predominantly divide asymmetrically to contribute to the growing retina. Apparently the mode of stem cell division is not a species-specific
feature, but rather characteristic for the type of stem cell niche and the function of the niche in tissue growth and/or homeostasis.
Footnote

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Competing interests
The authors declare no competing financial interests.

Author contributions
FG, NA and JW initiated the study and designed the experiments. FG, NA performed the experiments. FL, TB, TW, VW performed the micro-CT analysis, CQS did the MuViSPIM imaging and data analysis. LC contributed to lineage tracing experiments. FG, NA and J.W. wrote the manuscript with feedback from all co-authors.

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Figure 1. Medaka intestinal tract shows morphological and functional homology to the mammals

(A) 3D image of adult medaka taken by X-ray micro-CT. Anatomical landmarks are highlighted. Data were used for (B) reconstruction of the buccal cavity. (C) esophagus, B, C rostral to caudal perspective. (D) midgut; anterior: left with densely packed folds; posterior: right with elongated folds. (E) posterior gut; anterior: left; posterior: right. (F-I) H&E stained transverse sections of adult gut along rostro-caudal axis. Histology of intestinal folds in each segment in (F-I) shown in (J-M). Morphology of folds varies along rostro-caudal axis. (N) Gene expression of selected marker genes in six rostro-caudal segments of adult intestine. Control: elongation factor 1α. Note that apoal and fabp2 are only detectable in four rostral segments. Expression of large intestinal marker fabp6 confined to caudal segments S3 to S6. Expression of.ctsl1 in segments S5, S6 (N). Schematic summary of RT-PCR results shown in (O). Abbreviations: b, brain; bc, buccal cavity; bv, blood vessel; e, enterocyte; g, gut; gi, gills; h, heart; l, liver; lp, lamina propria; msc, mucuse secreting goblet cells; n, notochord; o, operculum; oe, oesophagus; ov, ovary; pef, pelvic fin; pf, pectoral fin; sb, swim bladder; s, spinalcord; t, thymus; tm, tunica muscularis; tp, tongue papilla-like; ts, tunica serosa; va, ventral aorta. Scale bar for F-I 200 µm and for J-M 25 µm.
Figure 2. Stem cell marker genes expressed at base of intestinal folds in the adult intestine

RNA *in situ* hybridization of markers on sections of the medaka adult intestine, anterior and midgut. (A-C) Graded expression of *lgr4*, *lgr5* and *lgr6* from base of to centre of intestinal folds. (D-F) Weak *bmi1*, *bmi3* and *aldh1a* expression, with more prominent *aldh1a* in *lamina propria* and supporting tissues. (G) *ascl1a* expression restricted to the patches. (H-I) *rspo1* expression within fold, while *rspo3* is confined to underlying connective tissue. (J-L) *axin* (*axin2a* and *axin2b*) and *sox9b* show clear expression confined to base of the fold. Scale bar 50µm.
Figure 3. Expression pattern of intestinal \textit{sox9} is conserved from Lamprey to medaka

(A) Transverse section of lamprey larval intestine stained with haematoxylin and eosin highlighting the typhlosole as single fold. 
(B) \textit{In situ} detection of \textit{Sox9} expression at base of typhlosole (T). 
(C) EdU positive cells (24 hour after injection) reveal basal proliferation zone.
(green, nuclei: DAPI, blue). (D) medaka Sox9b:eGFP-expressing cells mark proliferative intestinal cells at base of furrows. Confocal images of transverse cryosections of sox9b:gfp transgenic intestine at 10 dpf. (E) three-week-old juveniles (F) and eight-week-old adult fish. Yellow arrows indicate position of sox9b:eGFP-expressing cells at base of folds. (G) Co-localization of endogenous sox9b expression domain shown by in situ hybridization and proliferation in EdU+ cells. (H) Co-localization of sox9b:eGFP-expressing cells and EdU staining. Arrows indicate sox9b:eGFP-expressing cells. Note that sox9b:eGFP-expressing cells are EdU positive. (I) Sox9b endogenous expression shown by in situ hybridization in Gaudi\textsuperscript{RSG-ubiquitin\textsuperscript{ERT2}}Cre transgenic fish (left panel), clonal analysis of Gaudi\textsuperscript{RSG-ubiquitin\textsuperscript{ERT2}}Cre transgenic fish two weeks after induction. At base of furrow proliferating GFP+ stem cells (middle panel) express sox9b (right panel). Scale bars 100 µM (A-C); 25 µm (D,G,H,I); 50 µm (E,F). BV, blood vessel; I, intestine; T, typhlosole.
Figure 4. Intestinal stem cells located in furrows between intestinal folds

(A) pH3-immunostaining on transverse sections of fold. Fold was divided into four equally sized regions: Base (B), middle-base (MB), middle-tip (MT) and tip (T). Arrows in left panel indicate pH3 positive cells. Respective frequency of pH3 positive cells shown in right panel. Majority of pH3 positive cells at base of fold. (B) Caspase-3 immunostaining on transverse sections of a fold. Caspase-3+ cells located at fold tip. (C, D) EdU pulse-chase assay. Adult fish were incubated in EdU for 24hrs and fixed after 12, 36, 50, 122hrs. For each time point, a representative fold from anterior and mid gut is shown in (C). Frequency of EdU+ cells in each region (D) was counted and analysed with KNIME. For each time point three fish were analysed and nuclei from around 50 folds were counted for each region. Scale bar 50 µm.
Figure 5. Clonal analysis in the Gaudi^{RSG} line indicates preferred symmetric cell division in the medaka intestine

(A) Experimental timeline. Double transgenic fish Gaudi^{RSG}-ubiquitin^{ERT2}Cre were employed. Inducible Cre recombinase triggers shift in reporter colour and localization. Cre-mediated recombination of Gaudi^{RSG} construct was triggered at larval stage by tamoxifen treatment (5-10µM, three hours). Green arrows indicate time point of imaging and analysis of the intestine after the induction. (B) Whole mount representation (macroscope) of...
dissected intestines at different time points (B) one day (C) 10 days (D) 30 days (E) 150 days post induction. (B’-E’) Confocal images of intestinal sections of corresponding fish. Labelling of discrete, single cells at one dpi, larger clonal strings extending from bottom to top of folds (10dpi) and coverage of entire folds with descendants of individual recombined (or non-recombined, 30dpi, 150dpi) cells. Each panel represents a 3D projection of 60-100 optical sections (plane=0,5µm) H2B-GFP green, DAPI stained DNA (blue). Scale bar 50µm. (B’’-E’’) MuVi-Spim 3D visualization of gut segments (532µm) at different time point after induction showing labelling in the context of the organ. (F) GFP positive containing folds were counted using the sections of 19 fish one dpi, 11 fish 10dpi, 5 fish 30dpi, 6 fish 150dpi, unpaired t test, p value=0.0322. (G). Quantitation of clone size of intestinal segments shown in (B’’-E’’) at 10, 30, 150dpi, logarithmic scale, Mann-Whitney test, p value<0.0001. (H) Clone density per volume (mm³) of intestinal segments shown in (B’’-E’’). Cell numbers were derived from light sheet analyses, represented at logarithmic scale, Mann-Whitney test, p value=0.0005.
Figure 6. Clonal cell lineage tracing using Gaudi\textsuperscript{LxBBW} line confirms mode of cell division in the medaka intestine

A) Experimental timeline. Double transgenic fish Gaudi\textsuperscript{LxBBW} hsp70:nlsCRE were employed. Temperature shift inducible Cre recombinase triggers stochastic shift in reporter colour and localization. The shift was triggered at larval stage (12dpf) and intestines were analysed 90 dpi as indicated by green arrow. (B) Representation of multicolour Gaudi\textsuperscript{LxBBW} intestinal segment. Note that recombination in the tandem array of the LxBBW cassette results in multiple combinations of colours and localization, unambiguously barcoding each individual cell. (C). High resolution MuVi-Spim visualization (false colour) of intestinal segment (532\textmu m) showing multi-colour labelling in the context of the organ.
Figure 7. Model of ISCs in adult medaka fish

Bulging of the medaka intestinal epithelial surface creates folds and furrows. Proliferatively active intestinal epithelial stem cells located in furrows at base of intestinal folds. Apically adjacent, EdU+/pH3+ domain represents transit-amplifying compartment. Differentiated cells are pushed towards fold tip, where they are shed off. Lgr4/5/6, axin2a, axin2b, rsps1, stem cell markers, show graded expression in intestine at base of folds up to mid-base part. Sox9b expression confined to base of folds.
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Supplementary information

Figure S1:

(A) The anterior part of the medaka digestive tract. The x-ray tomography shows gall bladder and an attaching point of the liver via bile duct and gall bladder to the anterior part of the intestine tract, indicating a duodenum equivalent in Medaka. (B) Longitudinal section of adult Medaka esophagus analysed by MF20 immunohistochemistry demonstrating esophageal muscles by red color and nuclear staining by DAPI. (D) Alcian blue staining was used to visualize the mucus secreting Goblet cells in (D) most anterior part of digestive tract and (E) in mid gut. tm, tunica muscularis; msc, mucus secreting cells. Scale bar: 50μm
Figure S2:

Phylogenetic analysis of medaka lgr genes. The amino acid sequences of medaka (OL, *Oryzias Latipes*) LGR were compared to human (Hs, *Homo sapiens*), zebrafish (Dr, *Danio rerio*), mouse (Mm, *Mouse musculus*) and Xenopus (Xl, *Xenopus laevis*). For LGR5 the partial sequence available in NCBI has been considered (NCBI Reference Sequence: XP_004085697.2)
Movie 1: The rostral part of medaka intestinal tract.
A 3D view of X-ray data starting from buccal cavity and going through the mid-gut reveals distinct topographical regions along the rostral-caudal axis.

http://dx.doi.org/10.5061/dryad.591gf/1

Movie 2: The caudal part of the medaka intestinal tract.
A 3D view of X-ray data from the hind-gut indicating the narrower passage in the caudal part of the intestine with shorter folds.

http://dx.doi.org/10.5061/dryad.591gf/2

Movie 3: The GaudeoRSGubiquitin:ERT2Cre (10dpi)
MuVi-Spim 3D visualization of a gut segment at 10 days after induction shows already labeling of strings of clonally related groups representing the stem cells and their descendants (green).

http://dx.doi.org/10.5061/dryad.591gf/3

Movie 4: The GaudeoRSGubiquitin:ERT2Cre (30dpi)
MuVi-Spim 3D visualization of a gut segment at 30 days after induction showing intestinal folds that are either broadly labelled by H2B-EGFP (green), or not at all (grey) indicating longitudinal expansion of the clones.

http://dx.doi.org/10.5061/dryad.591gf/4
Movie 5: The Gaudi\textsuperscript{RSGubiquitin:ERT2Cre} (150dpi)

MuVi-Spim 3D visualization of a gut segment at 150 days after induction. Two large clones (in green) established by symmetric division and neutral drift.

http://dx.doi.org/10.5061/dryad.591gf/5

Movie 6: The Gaudi\textsuperscript{RBW hsp70:nlsCRE} (90dpi)

MuVi-Spim 3D visualization of a gut segment 90 days after induction shows that stochastically triggered clonal multi-color labeling results in adjacent clones of different sizes discernible by different fluorescent proteins demonstrated in this movie by yellow, green, blue and purple color.

http://dx.doi.org/10.5061/dryad.591gf/6