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Spatial and temporal expression patterns of chitinase genes in developing zebrafish embryos

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ABSTRACT

Chitinases and chitinase like proteins play an important role in mammalian immunity and functions in early zebrafish development have been suggested. Here we report identification of six zebrafish chitinases and chitinase like proteins (called CHIA.1–6) belonging to the glycoside hydrolase family 18, and determine their spatial and temporal expression at 10 stages of zebrafish development.

CHIA.4 is highly maternally expressed and it is expressed 100 fold above any other CHIA gene at zygote through to blastula stage. Later, after the maternal to zygotic transition, *CHIA.4* expression decreases to the same level as *CHIA.5* and *CHIA.6*. Subsequently, *CHIA.1*, *CHIA.2*, *CHIA.3* and *CHIA.4*, *CHIA.5*, *CHIA.6* each follow distinct paths in terms of expression levels.

Until 4 days post fertilization the spatial expression patterns of all six CHIA genes overlap extensively, with expression detected predominantly in vascular, ocular and intestinal tissues. At 5 days post fertilization *CHIA.1*, *CHIA.2* and *CHIA.3* are expressed almost exclusively in the stomach, whereas *CHIA.4*, *CHIA.5* and *CHIA.6* are also prominently expressed in the liver. These different expression patterns may contribute to the establishment of a basis on which functional analysis in older larvae may be founded.

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1. Results and discussion

Chitinases are enzymes that hydrolyse the ß-1,4 glycosidic bond of chitin. Chitin functions as a structural biopolysaccharide and is produced in vast amounts by fungi, insects, helminths, crustaceans and several other organisms (Jeuniaux and Voss-Foucart, 1991). Based on amino acid similarities chitinases have been divided into two groups, the glycoside hydrolase families 18 and 19 (GH18 and GH19), which employ different mechanisms to hydrolyse chitin (Henrissat, 1991). The defining feature of the GH18 family is the presence of the GH18 domain, which exhibits a TIM barrel fold. GH18 proteins hydrolyse chitin through a two-step general acid mechanism with substrate participation through an oxazolinium ion intermediate. The chitinolytic activity of the GH18 domain is critically dependent on a specific Glutamate residue, from here on denoted Glu_{cat}, which serves as the catalytic proton donor (Synstad et al., 2004; Van Aalten et al., 2001). The GH18 domain is often flanked by a smaller chitin binding domain, which in mammalian chitinase proteins is another hallmark of retained endochitinolytic activity. This domain exhibits six conserved Cysteine residues believed to form three disulphide bridges (Elvin et al., 1996).

In vertebrate genomes, the GH18 encoding genes generally fall into 3 groups, chitinase domain containing (CHID) genes, chitobiose (CTBS) genes and the chitinases/chitinase like proteins (CLPs), distinguishable by well conserved exon/intron structures. The former two are usually represented by one gene, while chitinases/CLPs are usually represented by several genes per genome.

The chitinase/CLP group include the genes encoding the two active endochitinases in mammals, the chitotriosidase (CHIT1) and the acidic chitinase (CHIA) (Bussink et al., 2007). In addition, mammalian genomes also encode a variable number of inactive CLPs with high sequence homology to the endochitinases. The CLPs have lost hydrolytic activity due to amino acid changes in their GH18 domain as well as loss of the associated chitin binding domain.

Mammalian chitinases/CLPs have been implicated in several different innate and acquired immune responses, as well as tissue remodelling and fibrosis, initiated by both chitinous and non-chitinous stimulants (Johansen, 2006; Lee et al., 2009; Malaguarnera et al., 2006; Reese et al., 2007; van Eijk et al., 2005; Zhu et al., 2004). In developing zebrafish larvae, chitin oligomers and the activity of one or more, as yet unidentified, chitinase(s), have been implicated in the normal development of trunk and tail (Bakkers et al., 1997; Semino and Allende, 2000).

Human CHIT1 was first discovered in the so-called Gauchers cells, which are lipid laden macrophages (Hollak et al., 1994). Expression of the CHIT1 gene has since been found upregulated in several other disease conditions in pathologic activated tissue

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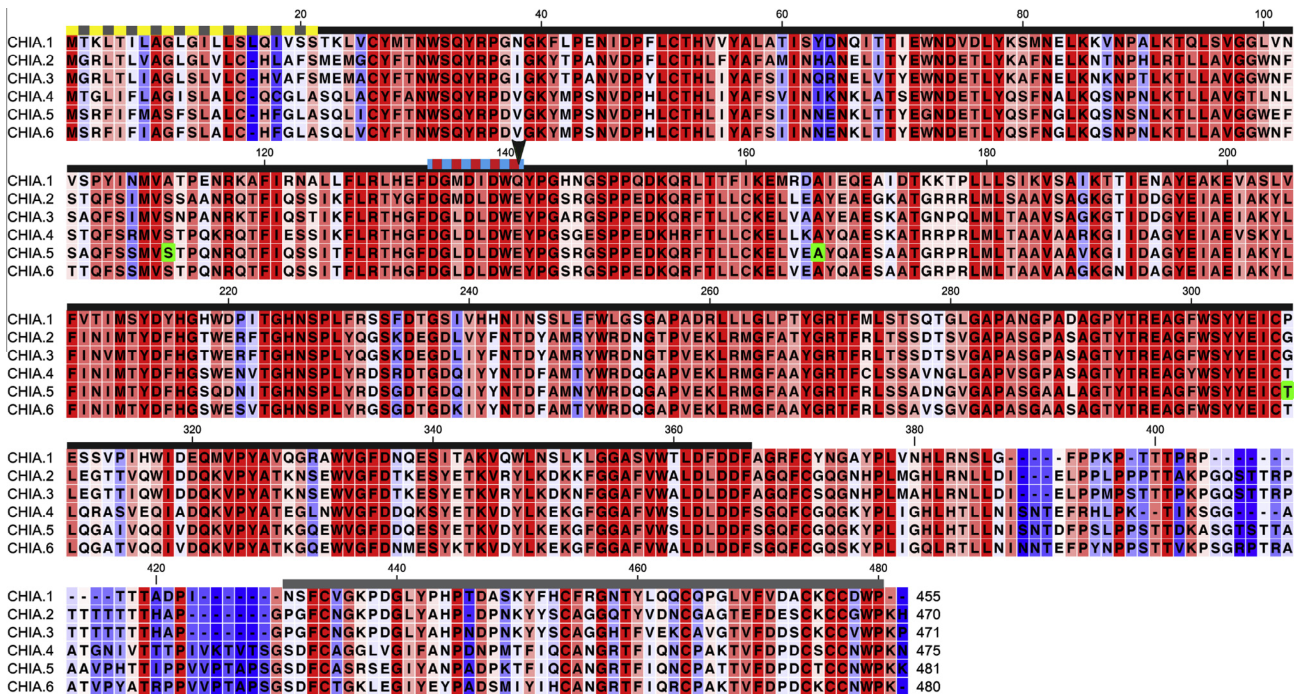

Fig. 1. High degree of conservation of sequence and important features of CHIA proteins. Alignment of the deduced amino acid sequences of the zebrafish CHIA genes. The degree of conservation is depicted in shades from blue to red, blue representing least conservation. The yellow-and-grey bar above the C-terminal most part of the alignment denotes amino acids constituting a signal peptide, as predicted by the signalP online tool (http://www.cbs.dtu.dk/services/SignalP/). The black and grey bars mark the GH18 and chitin binding domains, respectively, as predicted by the SMART tool at EMBL (http://smart.embl-heidelberg.de/). The red-and-blue bar and arrowhead marks the position of the active site and Glu_{cat} residue. Only the CHIA.1 protein lacks the Glu_{cat} residue and can be regarded as a CLP, though it retains a recognizable chitin binding domain. Marked with green squares at positions 113, 165 and 307 of *CHIA.5* are amino acids that, in our sequencing of the CDS, were detected as non-synonymous variances to the published gene sequences. The published sequence encodes a Serine in position 113, a Threonine at position 165 and an Asparagine at position 307 (refer to NCBI accessions: AAI53463.1 and NM_001110041.1).

macrophages, e.g. in atherosclerotic plaques (Boot et al., 1999) and Kupffer cells in non-alcoholic fatty liver disease (Malaguarnera et al., 2006). In mice *CHIT1* is expressed in the lung (Gavala et al., 2013), gut (Ohno et al., 2012), eye and lacrimal gland (Hall et al., 2008). In all tissues expression seems to be specific to activated macrophages and neutrophils (Boot et al., 1995; Boussac and Garin, 2000; Malaguarnera et al., 2006). CHIT1 is believed to function as an antifungal agent (van Eijk et al., 2005) complementing lysozyme in antimicrobial defence (Hall et al., 2008).

The second active mammalian chitinase discovered was the acidic mammalian chitinase (CHIA), named according to its remarkably low functional pH optimum (Olland et al., 2009). *CHIA* is also expressed in the gut and lungs of mice (Boot et al., 2001; Ohno et al., 2012). *CHIA* is upregulated in allergic conditions such as allergic rhinitis and conjunctivitis (Cho et al., 2010; Musumeci et al., 2008) and has attracted new interest after its involvement in the pathology of asthma was uncovered (Zhu et al., 2004). CHIA is currently viewed as an important modulator of TH2 immune responses.

CHI3L1, a CLP found in all mammals, is expressed in the musculoskeletal system of developing human embryos in rapidly dividing and actively differentiating cells, such as osteogenic cells (Johansen et al., 2007). Chondrocytes and phagocytes have also been reported to express the CHI3L1 gene (Boussac and Garin, 2000; Hakala et al., 1993). Furthermore, studies have reported regulation of *CHI3L1* expression in colonic epithelial cells under inflammatory conditions of the intestine (Mizoguchi, 2006). The primary function of this CLP is believed to be tissue remodelling, cellular mitogenic signalling and angiogenesis (Tang et al., 2013; Kawada et al., 2012).

The evolution of mammalian chitinase and CLP genes has been the focus of several studies (Bussink et al., 2007; Funkhouser and Aronson Jr., 2007; Hussain and Wilson, 2013). It has been suggested that an ancient gene duplication most likely led to the emergence of the *CHIT1* and *CHIA*, which have since undergone functional specialization. Later duplications to each of these genes, with loss of function mutations, led to the different CLPs (Bussink et al., 2007). A more recent study of GH18 gene evolution in a broader group of vertebrate genomes came to the same conclusion. Here the authors included zebrafish and speculated that the one zebrafish gene could be an ortholog of the mammalian CHIT.1 genes while the other zebrafish chitinase/CLP genes were *CHIA* paralogs. In the same study the authors speculate that the numerous *CHIA* paralogs have arisen from the teleost specific whole genome duplication known as 3R WGD (Hussain and Wilson, 2013). The products of these genes may since have acquired distinct functions rather than slowly eroding, which has been the fate of several other redundant genes produced by the 3R WGD (Brunet et al., 2006).

In spite of their recognized broad impact on immune responses in mammals, and the possible involvement in early embryonic development of trunk and tail in zebrafish (Semino and Allende, 2000), information about chitinases and CLP gene expression is scarce. Likewise, there are no comprehensive studies of their expression patterns in developing vertebrates. Such a study might shed light on possible developmental functions of the chitinase and CLP gene products. Here we report the expression patterns of six genes encoding chitinases/CLPs at 10 different stages of embryonic development in zebrafish larvae, in order to provide a base for future elucidation of the physiological functions of vertebrate chitinases/CLPs.

1.1. Identification of zebrafish GH18 genes

Our mining of the ZFIN genome database revealed a total of six chitinase/CLP encoding genes, named *CHIA.1* to *CHIA.6*

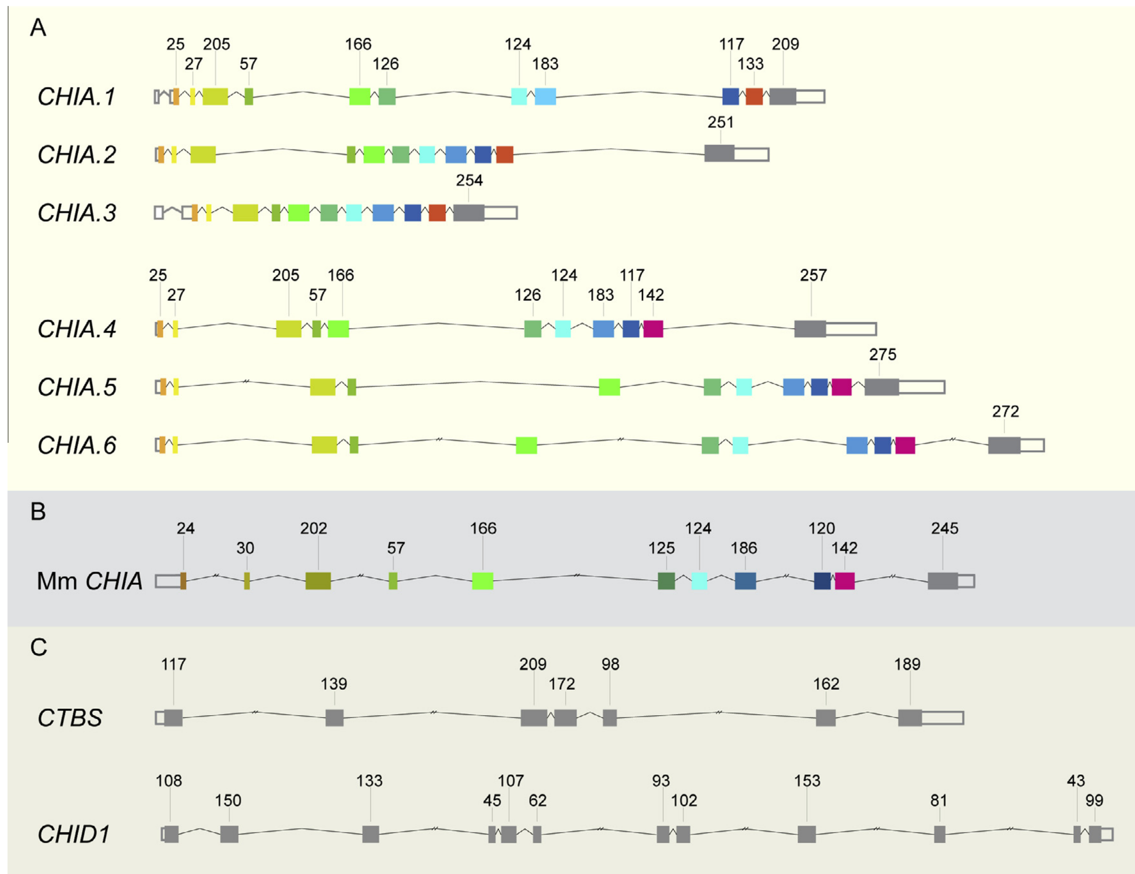

Fig. 2. The exon/intron structure of zebrafish CHIA genes clearly distinguishes them from the CTBS and CHID1 genes, and groups them with the chitinase/CLP group of GH18 genes. (A) The six zebrafish chitinase/CLP genes all share an almost completely conserved exon/intron structure. Exons retaining the same number of coding nucleotides are coloured accordingly. Only the last exon does not show this high degree of conservation and is coloured grey. The lengths of the penultimate exons separate the CHIA.1, CHIA.2 and CHIA.3 genes from the CHIA4, CHIA.5 and CHIA.6 genes. (B) Schematic representation of the mouse Chia gene. The number of coding exons and also, with some approximation, their length is conserved between zebrafish and mouse CHIA. (C) The zebrafish CTBS and CHID1 genes are clearly different from the CHIA genes in terms of exon/intron structure. The figure is based on the graphical output of the ENSEMBL Zv8 genome browser (http://aug2010.archive.ensembl.org/Danio_rerio/Info/Index).

(see Supplemental Fig. 5 for the previous ZFIN annotations). To validate the genes and the gene structures cDNA was produced from purified total RNA and sequenced. The corresponding exons were aligned to published genome sequences. *CHIA.5* was the only CHIA gene to exhibit non-synonymous nucleotide variations in the coding sequence (CDS) (see Fig. 1). As shown in Fig. 2 the characteristic exon/intron structure of the mammalian chitinase/CLP genes are conserved in all 6 zebrafish genes. These six chitinase/CLP genes, encode proteins that share the canonical domain structure of chitinases with a highly conserved GH18 domain, followed by a chitin binding CHTBD2 domain. While mammalian genomes encode only 2 active chitinases and a variable number of CLPs, five out of the six identified zebrafish CHIA genes are predicted to encode active chitinases, based on the retained Glu_{cat} residue in their deduced amino acid sequences and the presence of a recognizable chitin binding domain (see Fig. 1). Interestingly *CHIA.1* encoded protein and a predicted protein from Medaka are the only examples of CLPs retaining a chitin binding domain.

Like all other vertebrates, the zebrafish genome also encodes a CTBS and a CHID protein, both of which lack the chitin binding domain (Fig. 2).

While the 8th version of the ensemble genome browser had all six CHIA genes localised in two distinct clusters on chromosomes 11 and 23, the 9th version has only the CHIA.3, CHIA.4 and CHIA.6 genes annotated in the genome. Closer inspection shows that *CHIA.5* can still be found in the genome but has been annotated as a transcript of *CHIA.4*. Our data shows that this annotation is incorrect, since our cloning and sequencing of the *CHIA.4* and *CHIA.5* clearly identifies them as separate genes, with just under 87% identical CDSs.

We have cloned the CDS for all six CHIA genes from total RNA derived from zebrafish tissue and validated the sequences, positively proving the existence and active transcription of all six zebrafish CHIA genes. The disappearance of *CHIA.1* and *CHIA.2* from the ensemble Zv9 genome browser may be due to overly stringent filtering.

1.2. Staged embryonic expression of zebrafish CHIA genes

In humans and mice, two chitinases and several CLPs have assumed distinct functions and expression patterns. The retention of six highly homologous CHIA genes in the zebrafish genome might suggest a similar degree of functional specialization and diversification of expression patterns. We have developed qPCR assays and antisense RNA probes to explore the patterns of expression exhibited by each of the six zebrafish CHIA genes at ten developmental stages, from zygote to the 5th larval day. Considering the high sequence homology, the least conserved transcript sequences were targeted to avoid cross reactivity of probes and qPCR assays. By strand specific RT PCR we have shown that several of the CHIA transcripts exhibit antisense transcription, therefore rather than using sense probe controls, two non-overlapping in situ probes were generated for each gene and only patterns exhibited by both probes are mentioned (see S3 for pictures of results of

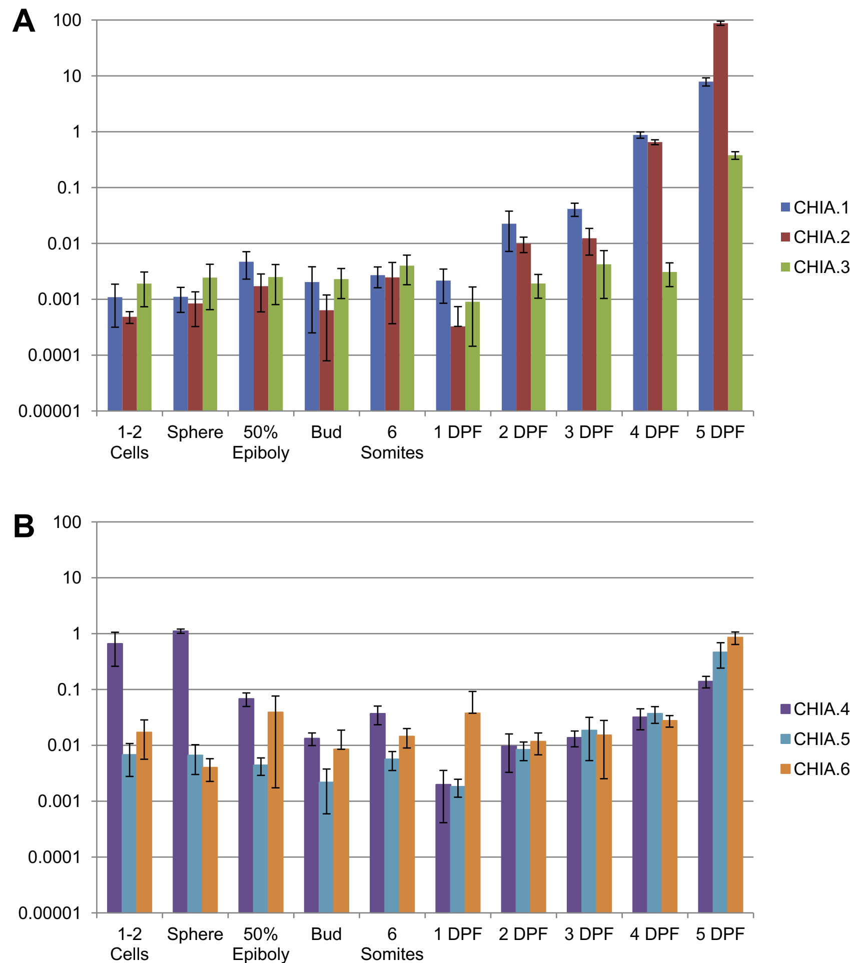

Fig. 3. Relative quantification of zebrafish CHIA gene expression at ten stages of development. qPCR measurements of gene expression are shown from the 1 to 2 cells stage until 5 days post fertilization. The house keeping genes RNA polymerase II and 18S ribosomal subunit were used to normalize the measurements. According to quantitative gene expression levels, the CHIA genes can be roughly divided into two groups: (A) *CHIA.1*, *CHIA.2*, and *CHIA.3* have very low levels of expression at the earliest developmental stages, but these rise sharply from day 3 to 4. (B) *CHIA.4*, *CHIA.5* and *CHIA.6* are more highly expressed at the earliest stages followed by a slower increase in expression. Interestingly, the *CHIA.4* has an apparent maximum at the sphere stage and expression decreases at later stages, after the maternal to zygotic transition. Error bars indicate standard deviations.

all probes at all stages and S4 for an overview of the evidence for the presence of antisense transcripts).

At the zygote and midblastula stages of embryonic development *CHIA.4* is expressed at a level 100 fold higher than the CHIA.5 and CHIA.6 genes, while *CHIA.1*, *CHIA.2* and *CHIA.3* are lower still. After peaking at the sphere stage, *CHIA.4* expression decreases to the same levels as *CHIA.5* and *CHIA.6* as development continues (Fig. 3A and B). Interestingly, the sharp decrease in *CHIA.4* expression, evident from the qPCR data (Fig. 3B), at the onset of gastrulation coincides with the maternal to zygotic transition (Baroux et al., 2008). From the 50% epiboly stage onward, the genes can be roughly divided into two groups based on their expression levels until 5 days post fertilization (DPF). *CHIA.1*, *CHIA.2* and *CHIA.3* are all very lowly expressed until 3–4 DPF and then rise significantly (up to 10.000 fold for *CHIA.2*). In contrast, *CHIA.4*, *CHIA.5* and *CHIA.6* are expressed at a higher and more stable level in the earliest developmental stages until the end of the segmentation period and then increase less markedly at 4–5 DPF (Fig. 3A and B).

In situ hybridization based on digoxigenin labeled antisense RNA probes was used to obtain information on the cellular expression patterns of the zebrafish CHIA genes. No differential spatial pattern can be discerned for any of the CHIA genes expression at stages earlier than 1 DPF (Fig. 4A–E), and at these stages non-specific background cannot be excluded as the cause of some of the staining. At 1 DPF all the CHIA genes seem to be expressed mainly in the developing eye and brain and at the myotome borders along the tail (Fig. 4F and G).

At 2 DPF the CHIA genes are all expressed in the lens of the eye and the caudal aorta (Fig. 5A–C). Expression in some of the major blood vessels of the head, the primary head sinus and the middle cerebral vein, also seems detectable at this stage (Fig. 5A1–6 and B), however these signals are faint, and hard to assign with certainty. The emerging pectoral fin buds are visible in dorsal views of *CHIA.2*, *CHIA.5* and *CHIA.6* (Fig. 5A-2, A-5, A-6).

At 3 DPF CHIA genes still seems to be expressed in the vasculature. Expression of all CHIA genes is visible in caudal aorta and major blood vessels of the head (Figs. 5D1–6 and 6E). At this stage the CHIA genes are also expressed in the myocardial tissue of the heart, the intestinal bulb and the mesenchymal cells around the newly opened mouth, the pharynx and the pharyngeal arches (Fig. 5D1–6 and E). In the dorsal view expression in the inner plexiform layer and optic nerve of the eye, as well as the pectoral fin can be defined (Fig. 5D1–6, E and F).

4 DPF the vascular tissue no longer seems to express CHIA genes, only a faint staining is visible in the caudal aorta. The mesenchymal tissue expression around the mouth and myocardial expression seems to have ceased entirely and the pharyngeal arch expression has subsided substantially, though it is still visible

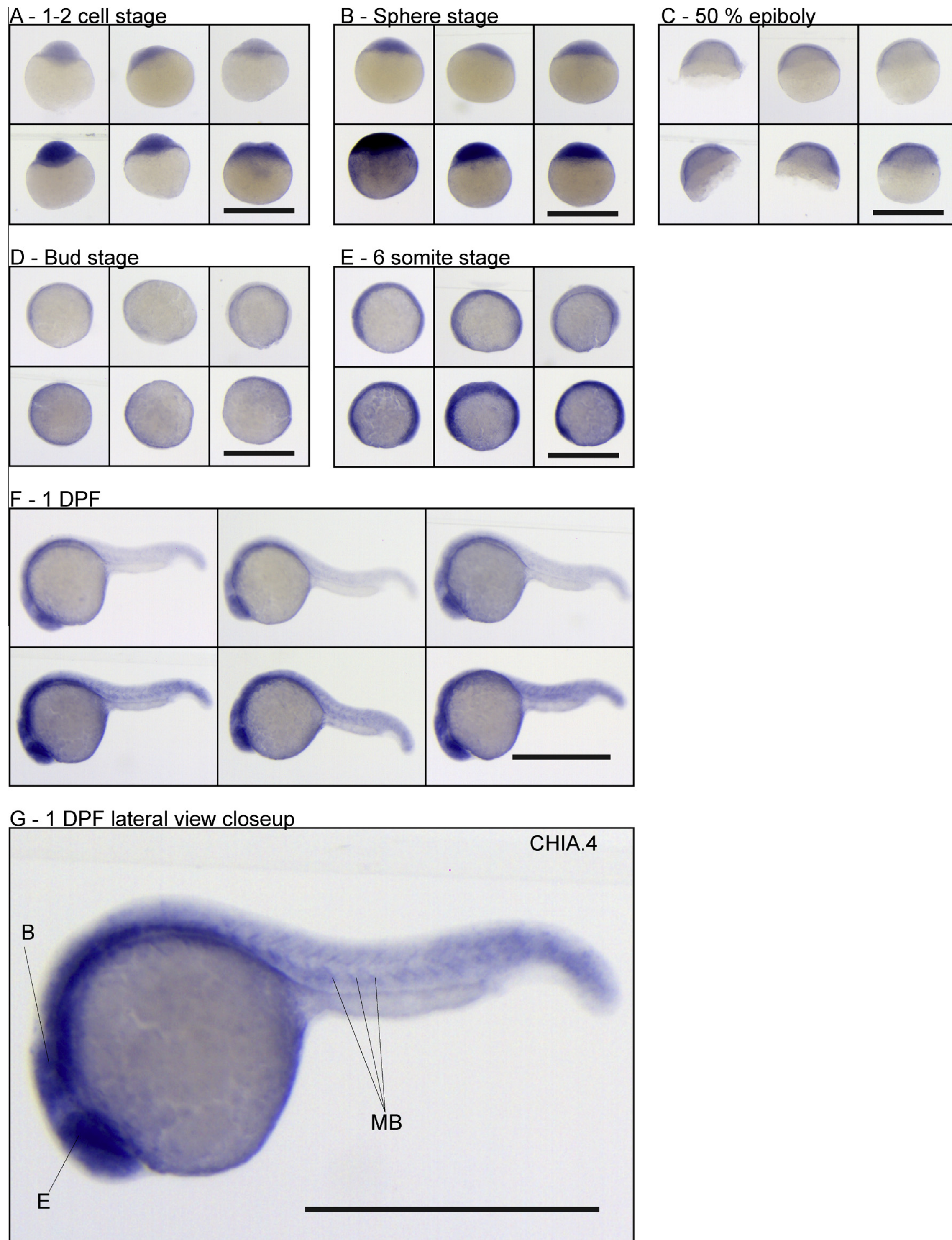

Fig. 4. CHIA gene expression from zygote to segmentation. (A–F) In situ hybridization results for the six earliest stages. In each panel the order is the same, top left: *CHIA.1*; top centre: *CHIA.2*; top right: *CHIA.3*; bottom left: *CHIA.4*; bottom centre: *CHIA.5*; bottom right: *CHIA.6*. The bar represents 1 mm in each case. (A–E) No differential pattern of gene expression can be distinguished in any of the initial stages of development. The early peak in *CHIA.4* expression (Fig. 3B) can be clearly seen in panels A and B. After sphere stage, all CHIA appear ubiquitously and lowly expressed (C–E). At 24 HPF the first distinguishable patterns can be seen (F). (G) Scale-up picture of the *CHIA.4* ISH result, representative for patterns visible in all ISHs: the signal is concentrated in the developing eye (E) and brain (B), as well as at the myotome borders (MB).

(Fig. 6A1–6). The white matter in the hind- and midbrain along with the intestinal bulb appear to be the major tissues of expression of all 6 CHIA genes (Fig. 6A1–6 and B). In microsectioned specimens the white matter signal can be clearly discerned defining the dorsal part of the hind- and midbrain in a layer of fairly uniform thickness. Also the pharyngeal arches and several head blood vessels are visible (Fig. 6C). *CHIA.4* seems clearly expressed in the habenulae at 4DPF, while for the other CHIA genes this pattern is less pronounced (compare Fig. 6A1–6 and D). The stomach is clearly becoming a major tissue for *CHIA.1* expression at this stage, while *CHIA.5* and *CHIA.6* are more highly expressed in the liver (Fig. 6A-1, A-5 and A-6).

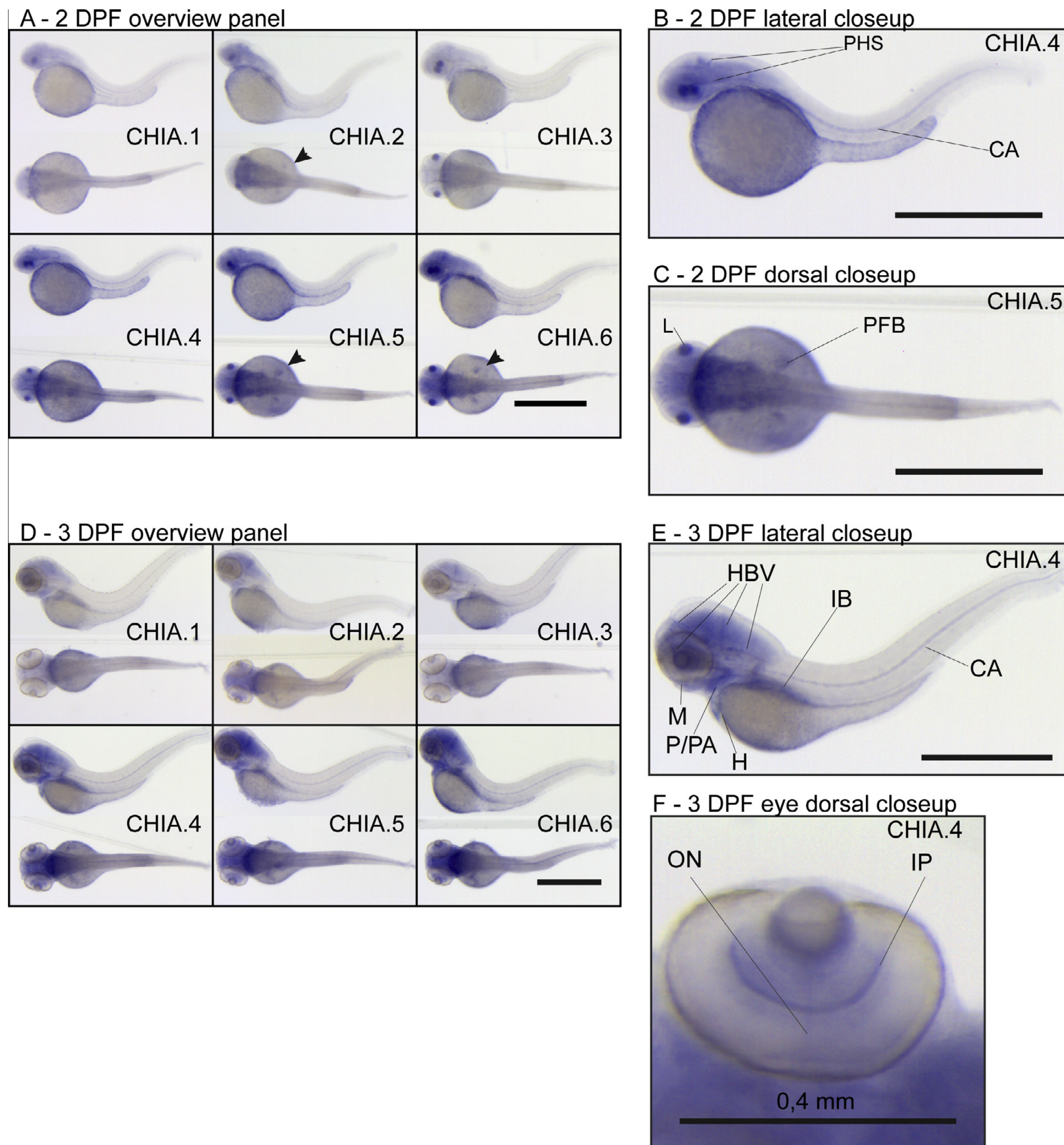

Fig. 5. *CHIA* expression patterns at pharyngula through to the early larval stages. (A–C) 2 DPF, (D–F) 3 DPF. The bar represents 1 mm except in F where it represents 0.4 mm. (A) 2 DPF panel of all CHIA (1–6) gene expression patterns. The lens of the eye and the caudal aorta seem to be the major tissues of expression of all the CHIA genes. The pectoral fin buds are visible in dorsal views of *CHIA.2*, *CHIA.5* and *CHIA.6* (arrowheads in A2, A5 and A6). (B) 2 DPF lateral close-up. The primary head sinus (PHS) can be seen as a curved line along the anteroposterior axis posterior to the eye. Extending dorsally immediately posterior to the tectum runs what appears to be the middle cerebral vein (MCV). Along the tail the caudal aorta (CA) can be seen extending all along the trunk and tail. (C) 2 DPF dorsal close-up. The lens (L) displays a strong signal at this stage. (D) 3 DPF panel of all CHIA gene expression patterns. (E) 3 DPF lateral close-up. The head blood vessels (HBV) can be seen at several places in the brain region. The tissue around the newly opened mouth (M), and the pharynx (P) and pharyngeal arches (PA) can be distinguished. The heart (H), caudal aorta (CA) and intestinal bulb (IB) also exhibit clear expression. (F) Dorsal close-up of the eye: the inner plexiform (IP) layer forms a clear cup shaped symmetrical pattern around the lens. Faint expression is visible in the optic nerve (ON) extending from the lens.

By 5 DPF the same rough grouping can be applied with regard to spatial expression patterns as could to the temporal development of gene expression (Fig. 3A and B), where the CHIA1, CHIA.2 and CHIA.3 genes follow a path distinct from that of the CHIA.4, CHIA.5 and CHIA.6 genes. The CHIA.1, CHIA.2, and CHIA.3 genes are almost solely expressed in the stomach lining with only very minute apparent expressional contributions from other tissues (Fig. 6D-1, D-2 and D-3). In contrast, the rise of *CHIA.4*, *CHIA.5*, and *CHIA.6* overall expression levels are to a higher extent due to an increased expression in the liver and only to a lesser extent due to expression in the stomach. The liver seems to be the main tissue for *CHIA.6* expression (Fig. 6D-4, D-5 and D-6). *CHIA.5* and *CHIA.6* expression in the head blood vessels is still detectable, but not in the caudal aorta. It should, however, be mentioned that at this stage some tissues, primarily in the trunk and tail, are hardly permeable to the probe, so the absence of ISH signal is not necessarily indicative of absence of expression.

1.3. Concluding remarks

Here we report the identification of six zebrafish genes that clearly belong to the chitinase/CLP group. We have cloned and sequenced all of these CHIA genes from total RNA and verified their sequences. Five are predicted to encode active chitinases according

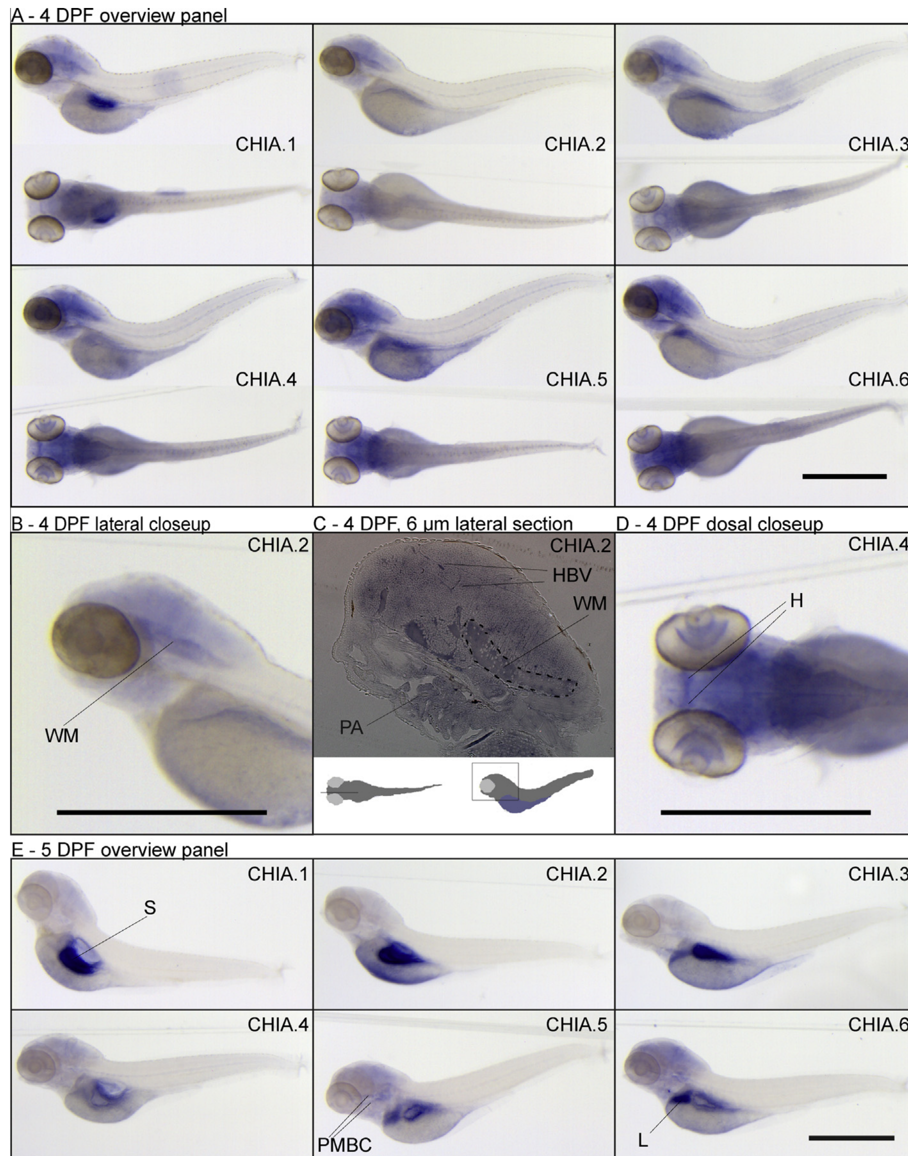

Fig. 6. *CHIA* expression patterns at the larval stage; days 4 and 5. (A–D) 4 DPF larvae, (E) 5 DPF larvae. All bars represent 1 mm. (A) 4 DPF panel of all CHIA gene expression patterns. Expression in intestinal tissues become increasingly visible and is especially clear in the stomach for *CHIA.1* (A1), and the liver for *CHIA.5* and *CHIA.6* (A5–6). (B) Lateral 4 DPF head close-up. The white matter (WM) can be distinguished clearly over the generally diffuse staining in the brain. (C) 6 μm section through the head of an embryo at 4 DPF. The white matter (WM) staining is outlined, and the pharyngeal arches (PA) and several head blood vessels (HBV) are also clearly discernable. Underneath is a schematic representation showing the approximate position of the slice in the embryo. (D) Dorsal 4 DPF head close-up. The habenulae (H) can be clearly distinguished in *CHIA.4*. (E) 5 DPF panel of all CHIA genes. *CHIA.1*, *CHIA.2* and *CHIA.3* (E1–3) are all nearly exclusively expressed in the lining of the stomach (S). *CHIA.4*, *CHIA.5* and *CHIA.6* much less pronounced so (E4–6). Some major blood vessels of the head are still visible such as the primordial midbrain channel (PMBC in E5). *CHIA.6* seems to be primarily expressed in the liver (L in E6).

to the presence of crucial domains and the Glu_{cat} residue in deduced amino acid sequence. Expression of each CHIA gene has been quantified at ten separate stages of embryonic development and we have assessed their tissue expression patterns.

CHIA.4 stands out as markedly more maternally expressed than any of the other CHIA genes. At the maternal to zygotic transition, at the onset of epiboly, expression decreases to a level similar to *CHIA.5* and *CHIA.6*. If indeed a chitinase, through some interplay with endogenous substrates, is implicated in the early embryonic developmental processes, as suggested by the studies of Bakkers and co-workers (Bakkers et al., 1997) and those of Semino and Allende (2000), CHIA.4 seems the strongest candidate for such a function.

After the early expressional peak the genes *CHIA.4*, *CHIA.5* and *CHIA.6* follow a similar path slowly rising in expression. *CHIA.1*, *CHIA.2* and *CHIA.3* follow a different path of expression; very low in the first developmental stages then rising abruptly from 3 to 4 (DPF). The differences between our observations and previous observations available as directly submitted data reports on the Zfin webpage (http://zfin.org/), which report no detectable expression of *CHIA.1*, *CHIA.2* and *CHIA.3* at stages prior to 5 DPF, are likely to arise from different experimental proceedings, especially differences in the length of the staining reaction.

The CHIA genes exhibit highly overlapping tissue expression patterns from the gastrula stage through to the hatching period. All six CHIA genes are expressed in ocular neuronal tissues from 1 to 3 DPF, in major vessels of the vascular system from 2 to 4 DPF and strongly in the white matter at 4 DPF. The tissues comprising the intestinal bulb clearly express CHIA genes from 3 to 5 DPF. At 4–5 DPF the first clear differences in expression

patterns becomes apparent, when *CHIA.1*, *CHIA.2* and *CHIA.3* are almost exclusively expressed by the lining of the stomach, while *CHIA.4*, *CHIA.5*, and *CHIA.6* exhibit a less pronounced stomach staining pattern, but a liver expression of equal or higher strength.

2. Experimental procedures

2.1. Zebrafish husbandry

Albino zebrafish were handled in accordance with animal welfare regulations as they apply at the local facilities in the Netherlands or in Denmark. Zebrafish were maintained according to standard protocols which can be found on http://zfin.org/.

2.2. RNA purification and cDNA synthesis

For all groups 20 embryos were snap frozen in liquid nitrogen and RNA extraction was performed using the trizol method (Invitrogen), in accordance with the suppliers' protocol. Contaminating DNA was degraded by DNase I treatment (Thermo-Fischer) and further purified by acid phenol:chloroform extraction. RNA integrity was assessed by a bioanalyser (Aglient) and only samples with an RNA integrity number (RIN) of 7 or higher were included in the analysis. cDNA was synthesised using the first-strand cDNA synthesis kit (ThermoFischer) using oligo dT priming.

2.3. qPCR

All qPCR experiments were carried out on a Roche Lightcycler 480, using SYBR green I mastermix (Roche, cat.-nr.: 04707516001). For each CHIA gene a panel of 4–6 primer pairs were tested and the best performing pair was further optimized in terms of annealing temperature, until efficiencies between 95% and 105% were achieved as assessed by a cDNA dilution curve. Further efficiency correction was performed by LinRegPCR analysis (Ramakers et al., 2003) on individual samples. All samples were collected in biological duplicate and each sample tested in technical triplicates. Geometric mean values were normalized according to the two house-keeping genes RNA polymerase II and 18S ribosomal subunit.

QPCR primer sequences (5′–3′):

CHIA.1 F: ACTGGGCGGAGCCTCAGTGT R: GGGCTTGGGTGGGAAACCCAG

CHIA.2 F: GGTGCTCTGCCACCTTGCCTT R: GGCATGGTTGATCATGGCGAAAGC

CHIA.3 F: TCGACCCTTACCTTTGCACACACCT R: ACACCATGATGGAGAACTGTGCCGA

CHIA.4 F: TGGACACCTCCACACGCTGC R: ATGCCCACTAATCCGCCCGC

CHIA.5 F: CCACGGCTCACAGGACAACATCA R: GTCCGCAGACGACAGGCGAA

CHIA.6 F: TCCACGGCTCATGGGAGAGTGTC R: AGCGCCCTGATCTCGCCAGT

HK1 (18S) F: TCGCTAGTTGGCATCGTTTATG R: CGGAGGTTCGAAGACGATCA

HK2 (RNApII) F: CCAGATTCAGCCGCTTCAAG R: CAAACTGGGAATGAGGGCTT

Reaction conditions: All qPCR reactions followed the same basic 3-step thermocycler program: 95 °C for 5 min then 40 rounds of 95 °C 10 s, 56–68 °C for 5 s, 72 °C for 10 s.

Annealing temperatures: CHIA.1: 67 °C, *CHIA.2*: 64 °C, *CHIA.3*: 65 °C, *CHIA.4*: 66 °C, *CHIA.5*: 65 °C, *CHIA.6*: 68 °C, HK1: 60 °C, HK2: 56 °C.

2.4. Probe synthesis

Two non-overlapping digoxigenin labelled antisense RNA probes were generated for each CHIA gene. Probes ranged from 270 to 450 nucleotides in length. See Supplemental Fig. 1 for charts of CHIA genes and probe sequences.

Templates were generated by PCR amplification on plasmid borne fragments using T7 extended primers (see Table S2 for primer sequences). RNA probes were generated by *in vitro* transcription from the T7 RNA promoter, incorporating DIG-11-UTP (Roche, cat.-nr.: 11277073910) nucleotides, using T7 RNA polymerase (Fermentas, cat.-nr.: EP0111). Probes were precipitated by EtOH according to the supplier's protocol (Roche) and redissolved in DEPC treated water.

2.5. In situ hybridization (ISH)

ISH were performed on 3–5 embryos per stage per probe. Each gene was tested by two non-overlapping digoxigenin labelled antisense RNA probes. The ISH protocol used was essentially identical to that published by Thisse and Thisse (2008). Only significant alteration: secondary antibody was diluted 1:4000 rather than 1:10,000.

Plastic embedded micro-sectioning was performed as previously described (Laroche et al., 2013).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gep.2014.01.001.

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